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The Effects on Foetal Development of Freezing Pregnant Hamsters (*Mesocricetus auratus*)

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WITH TWO PLATES

INTRODUCTION

IT has been known for many years that chilling at sensitive stages of embryonic or larval life profoundly affects the development of cold-blooded animals. For instance, the wing pattern of butterflies can be changed by cooling their pupae; the central European tortoiseshell, *Vanessa urticae*, treated in this way emerges in a form closely resembling the arctic species *V. polaris*. *Drosophila* and other insects cooled as larvae show bizarre characteristics, while sea-urchin and fish cooled as early embryos develop into monstrosities. Similar aberrations occur naturally as a result of genetic mutations and can be produced experimentally in these animals by X-rays and other physical and chemical agents applied at critical periods of development (see Ford, 1945; Goldschmidt, 1938). X-rays administered at critical stages in pregnancy may induce embryonic death or foetal deformities in mammals. Some of the monstrosities are resorbed, aborted, or stillborn; others survive birth (Russell, 1950, 1956; Russell & Russell, 1954 *a*, *b*; Wilson, 1954). Various hormones, drugs, toxic substances, and virus infections cause similar abnormalities (Gillman, Gilbert, & Gillman, 1948; Thiersch, 1952; Nelson, Asling, & Evans, 1952; Hicks, 1953, 1954; Giroud, 1954).

By contrast, comparatively little is known about the effects of cold on early mammalian development. Vidovic (1952, 1956) found that rats at all stages of pregnancy survived cooling to deep body temperatures between 15° and 20° C. and occasionally as low as 12° C. The majority gave birth to normal young. The sensitivity to hypothermia increased after the 14th day and particularly after the 16th day of pregnancy so that litter size was small and the proportion stillborn increased. One rat cooled on the 17th day of pregnancy gave birth to three deformed foetuses. Courrier & Marois (1953, 1954), using a slightly different technique, found that uterine haemorrhage occurred in rats cooled to body temperature between 16° and 20° C. on the 12th to the 18th day of pregnancy. The

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majority of foetuses died and were resorbed. On the other hand, rats were cooled daily to the same degree during the first 11 days of pregnancy without harm to the embryos although implantation was delayed, development retarded, and parturition postponed.

Pregnant mammals have not hitherto been cooled to body temperatures below about 15° C., because at that point breathing and heart-beats usually cease and the animals appear to be dead. Recently, however, adult rats and mice have been revived after lowering the body temperature almost to 0° C. and after respiratory and cardiac arrest (Andjus, 1951; Andjus & Smith, 1955; Andjus & Lovelock, 1955; Goldzveig & Smith, 1956). Mature golden hamsters have been resuscitated after supercooling to or freezing at body temperatures below zero and have survived long periods thereafter (Smith, Lovelock, & Parkes, 1954; Smith, 1956 *a*, *b*).

Pregnant hamsters have since been cooled and revived in the same way. The effects on foetal development of supercooling and of freezing the mother were referred to briefly at a recent conference (Smith, 1956*c*). The results of freezing will now be described in detail.

EXPERIMENTAL PROCEDURE

The experiments were carried out in three successive years between July and October, a season when young golden hamsters breed without special conditions in the Medical Research Council's colony at Mill Hill.

Animals

The females weighed 80–90 g. They were 8–12 weeks old and were having regular 4-day oestrous cycles as judged by occurrence of post-oestrous vaginal discharges of mucus and nucleated epithelial cells (Deanesly, 1938; Ward, 1946). They were paired with slightly larger males (110–120 g.) at about 5.30 p.m. on the 3rd day after the vaginal discharge. The following morning vaginal smears were examined for spermatozoa. When mating had been confirmed in this way the females were housed in individual cages and examined daily to eliminate those in which oestrus recurred.

Timing the start of pregnancy

Hamsters usually mate at night and ovulation and fertilization occur in the early hours of the morning (Austin, 1955). The duration of pregnancy was therefore assumed to be approximately 12 hours by noon on the day when spermatozoa were found. This rough method of timing the start of pregnancy was checked by killing controls at intervals throughout the 16 days of gestation. The embryos and foetuses were examined and preserved for reference in 10 per cent. formol saline. A few animals were allowed to go to term. The young were counted after 5 days and again when they were weaned.

Cooling to and freezing at body temperatures below 0° C.

Animals in which pregnancy had lasted approximately $\frac{1}{2}$–$15\frac{1}{2}$ days were cooled by the method described (Smith, 1956*a*). The body temperature was kept below 0° and they were allowed to freeze progressively in baths at −5° C. for 25–30 min. in the first series and for 45–48 min. in the second series of experiments.

Resuscitation

Hamsters were thawed and reanimated by warming the whole body with diathermy and by giving artificial respiration following the standard technique (Smith, 1956*b*).

After revival

The animals were weighed daily. Vaginal smears were examined for blood or for recurrence of the oestrous cycle. A few females were allowed to go to term and rear litters. The majority were killed when pregnancy had lasted approximately $13\frac{1}{2}$ days. The living foetuses were compared with controls of the same age. Resorbing foetuses were examined and counted. The specimens were fixed in 10 per cent. formol saline.

Calculation of the proportion of body-water frozen

The amount of ice formed was calculated by Lovelock's method (Lovelock & Smith, 1956) using the formula

$$P = 1 - T_1 / T_2,$$

where P is the proportion of water frozen,

T_1 is the freezing-point of the fluid within the animal,

T_2 is the final temperature reached in the colon at the end of freezing.

RESULTS

Litter size and number of young reared

Untreated hamsters. Ten females which had mated were kept in the same room with the experimental animals. One continued to have oestrous cycles. Two became pseudopregnant. Seven had litters on the 16th day. Two animals killed and ate the whole litter soon after birth. The other 5 hamsters had between 5 and 8 young each, but only reared 2 to 5 of the litter. The conditions of a busy experimental room were obviously not satisfactory because litters of 6–8 are regularly reared in the breeding colony.

Hamsters frozen for 30 min. at different stages of pregnancy. Hamsters frozen when pregnancy had lasted approximately $1\frac{1}{2}$, $3\frac{1}{2}$, $4\frac{1}{2}$, $5\frac{1}{2}$, $8\frac{1}{2}$, and $12\frac{1}{2}$ days gave birth on the 16th day and had litters varying in size from 3 to 9. They reared between 2 and 8 young respectively.

Animals frozen at $9\frac{1}{2}$, $10\frac{1}{2}$, and $11\frac{1}{2}$ days had a blood-stained vaginal discharge during the 2 days after resuscitation and did not have litters.

The hamsters which were $13\frac{1}{2}$, $14\frac{1}{2}$, and $15\frac{1}{2}$ days pregnant had prolonged parturition lasting 6–24 hours, and then died. Several young were born alive but did not survive. At autopsy macerated foetuses were found in the uterus in each animal.

Hamsters frozen at $2\frac{1}{2}$, $6\frac{1}{2}$, and $7\frac{1}{2}$ days of gestation were either pseudopregnant or else they aborted or had litters which they destroyed before the young could be counted or examined. The results showed that freezing at some stages was consistent with normal development but suggested that, under the conditions available, it would be more informative to kill the mothers before parturition so as to examine the foetuses and placentae.

The numbers of eggs implanted and the chronology of development in untreated hamsters

Hamsters were killed on each day of pregnancy. The average number of recent ovulation points or corpora lutea in the ovaries was ten. The development of eggs during the first 4 days was examined by Dr. Austin and has been described elsewhere (Austin, 1955). Implantation sites were difficult to see with the naked eye at $4\frac{1}{2}$ days. The stages of development reached by embryos and foetuses whose ages were determined as described above (see p. 312) corresponded with Boyer's accurately timed observations (Boyer, 1948, 1953) showing that, for the experiments in hand, it was not essential to know the exact time when the parents mated. The number of implantation sites varied from 5 to 13 and averaged 9. There were one or two foetuses undergoing resorption in most litters on and after the 10th day of gestation. There were no striking differences other than size in the external appearance of foetuses between $13\frac{1}{2}$ and $15\frac{1}{2}$ days of age. More information about the numbers of foetuses and the stage of development at $13\frac{1}{2}$ days was therefore collected.

The numbers and appearance of foetuses at $13\frac{1}{2}$ days of gestation

Normal untreated hamsters. Fifteen hamsters were mated and 11 immediately became pregnant. One animal aborted on the 9th day of pregnancy and was set aside. Three others were pseudopregnant after mating for the first time but became pregnant after a second mating. Fourteen animals were killed after $13\frac{1}{2}$ days of gestation. There were 111 normal and 15 foetuses undergoing resorption. One foetus was undersized and slightly retarded in development. No other abnormalities were seen. The individual and average sizes of litters are shown in Table 1.

The hamster foetus aged $13\frac{1}{2}$ days shows definite features which distinguish it from specimens $\frac{1}{2}$ to 1 day younger. It moves actively for several hours *in vitro*. The head is held erect so that the chin and neck show up well. The back of the head is gently curved and does not show the angulation and flattening seen at

12½ days. The mouth is slightly open but the tongue does not protrude. The pinna is folded over the external auditory meatus. The eyes are closed except for a narrow slit at the top. The skin is wrinkled and is roughened by hair follicles over

TABLE 1

The numbers of normal and of resorbing foetuses in litters from untreated hamsters killed at 13½ days of gestation

Animal no.	*Number of foetuses*		
	Normal	*Abnormal*	*Resorbing*
14 . .	7	0	0
15 . .	8	0	0
28 . .	9	0	5
43 . .	8	0	2
65 . .	9	0	0
71 . .	13	0	1
40 . .	9	0	0
73 . .	5+1 undersized	0	2
75 . .	8	0	0
38 . .	12	0	1
76 . .	8	0	1
72 . .	5	0	0
77 . .	9	0	2
TOTAL .	111	0	14
Average	8	. .	1

the whole surface of the trunk. The umbilical cord is constricted near the abdominal wall and there is no sign of a hernia in this region. There are small claws on the digits of the fore and hind paws. These features are illustrated in Plate 1, fig. A.

Hamsters frozen for 25–30 min. at different stages of pregnancy. Hamsters which had been pregnant for 1½–12½ days were cooled till breathing and heart-beats ceased and then frozen for 25–30 min. at – 5° C. They were resuscitated and kept until gestation had lasted approximately 13½ days when they were killed. Litter size and the proportion of resorbing foetuses was normal in mothers frozen at any time between 1½ and 8½ days of pregnancy or at 12½ days (see Table 2). There were no deformed foetuses. With one exception the individual foetuses showed the distinctive characteristics described above which are normally present at 13½ days. A typical litter is shown in Plate 1, fig. B. The exceptional foetus was one of nine in the litter from the animal frozen at 8½ days of gestation. It was undersized, and its development was retarded by about half a day as judged by the position and shape of the head, the half-open eyes, and absence of skin wrinkles and claws. There was a similar foetus in one of the control litters.

Freezing at 9½ days of pregnancy, on the other hand, resulted in resorption of

all but one foetus in each of two litters. These two survivors were alive and normally developed as compared with controls (see Plate 1, fig. C). Freezing at $10\frac{1}{2}$ days caused partial resorption of all the foetuses. The eye was recognizable but none of the special features either of a $10\frac{1}{2}$-day embryo or of a $13\frac{1}{2}$-day foetus or any intermediate stage were evident (Plate 1, fig. D). Freezing at $11\frac{1}{2}$ days was less destructive and three foetuses in one litter were alive. One was

TABLE 2

The numbers of normal and of resorbing foetuses in litters from hamsters frozen for 30 min. at different stages of pregnancy and killed at $13\frac{1}{2}$ days of gestation

Animal no.	*Duration of pregnancy when cooled*	*Number of foetuses*		
		Normal	*Abnormal*	*Resorbing*
	(*days*)			
23 . .	$1\frac{1}{2}$	11	0	0
54 . .	$2\frac{1}{2}$	8	0	0
47 . .	$3\frac{1}{2}$	9	0	2
45 . .	$4\frac{1}{2}$	0	0	0
4 . .	$4\frac{1}{2}$	10	0	2
56 . .	$5\frac{1}{2}$	8	0	0
37 . .	$6\frac{1}{2}$	5	0	4
43 . .	$7\frac{1}{2}$	10	0	2
49 . .	$8\frac{1}{2}$	8+1 slightly undersized	0	0
41 . .	$9\frac{1}{2}$	1	0	9
62 . .	$9\frac{1}{2}$	1	0	7
44 . .	$10\frac{1}{2}$	0	0	10
61 . .	$10\frac{1}{2}$	0	0	11
43 . .	$11\frac{1}{2}$	2+1 slightly undersized	0	4
40 . .	$12\frac{1}{2}$	5	0	0
16 . .	$12\frac{1}{2}$	8	0	2
TOTAL .	..	88	0	53
Average	..	5·5	..	3

undersized and also retarded; its head, which was bowed so that the chin pointed down, was flattened and angulated in contour, the tongue protruded, and the eyes were more than half open. The pinnae did not cover the external auditory meatus. Skin wrinkles, hair follicles, and claws were absent (Plate 2, fig. E). The other two foetuses were almost of the size and stage of development normally reached at $13\frac{1}{2}$ days. All the resorbing foetuses from mothers frozen at $9\frac{1}{2}$–$11\frac{1}{2}$ days of pregnancy were attached to placentae and surrounded by membranes. The foetal sacs contained variable amounts of dark altered blood. This was not ordinarily seen at sites of foetal resorption in control animals.

These observations showed that freezing for 30 min. during the first half of pregnancy and at $12\frac{1}{2}$ days was harmless to the foetuses. They also showed that there was a critical period in gestation between $9\frac{1}{2}$ and $11\frac{1}{2}$ days when freezing

the mother caused destruction of the majority of foetuses and that the damage involved intrauterine haemorrhage.

TABLE 3

The numbers of normal, abnormal, and resorbing foetuses in litters from hamsters frozen for 45 min. at different stages of pregnancy and killed at 13½ days

Animal no.	*Duration of pregnancy when cooled*	*Number of foetuses*		
		Normal	*Abnormal*	*Resorbing*
	(*days*)			
42	½	0	0	7 very small placentae
61	½	0	0	0
69	1½	0	0	0
13	1½	0	0	0
10	2½	0	0	0
70	2½	0	11	0
19	3½	0	0	0
25	3½	0	0	0
37	3½	0	0	0
4	4½	0	0	0
27	4½	0	0	0
2	5½	0	0	0
30	5½	0	0	4 placentae
31	6½	2 retarded	5	6
24	6½	0	3	5
18	6½	0	0	0
23	6½	0	0	10 very small placentae
7	7½	5 slightly retarded	0	0
5	7½	0	6	3
52	8½	0	7	5
3	8½	3	0	5
9	9½	0	0	0
33	9½	3	0	4
67	9½	1	0	6
12	10½	1	0	8
35	10½	0	0	9
36	10½	1+1 undersized	0	7
49	11½	4	0	5
1	11½	3	0	7
8*	12½	3	0	0
16	12½	5	0	1
22	12½	6	0	3

* Number 8 had only one uterine horn.

Hamsters frozen for 45 min. at different stages of pregnancy. Hamsters were cooled until circulation and respiration had ceased, frozen for 45 min. at − 5° C. and then resuscitated. They were ½–12½ days pregnant at the time of the experiment and were killed when gestation should have lasted 13½ days. The results (see Table 3) showed striking differences from those of freezing for 30 min. (Table 2). There were no normally developed foetuses in any of the mothers frozen during the first week of pregnancy. Every foetus in a litter of 11 from one animal frozen at 2½ days was alive but grossly malformed and also undersized (see Plate 2, figs. F, G, H). Monstrosities and severely retarded foetuses were also found in two litters from animals frozen at 6½ days of pregnancy (figs. I, J). There were either collections of very small placentae or else no signs of intrauterine implantation in other hamsters frozen during this period.

Freezing for 45 min. at 7½ or 8½ days of pregnancy was not invariably harmful. In one litter, however, the six living foetuses were undersized and deformed (Plate 2, fig. K). In another litter there were seven recognizable embryos developed only to the stage usually reached at 9 or 10 days, although the mother was killed, as usual, after 13½ days' gestation.

The results of freezing for 45 min. at 9½, 10½, and 11½ days of pregnancy were essentially the same as those of freezing for 30 min. Most of the foetuses were resorbed, but those which survived were normal or slightly retarded. 12½ days was the only stage of pregnancy at which freezing for 45 min. had no serious effects on foetal development.

The most interesting findings in these experiments were the grossly abnormal foetuses, of which there were 32 from five mothers. The deformities are illustrated in Plate 2, figs. F–K. Oedema and haemorrhagic patches were present in most of these foetuses. The majority also showed abnormalities of the nervous system including hydrocephalus, anencephaly, and herniation of the brain on the top of the head. In some instances the whole brain was on the surface; in others there was a small protuberance or bleb. In addition, several foetuses had spina bifida or were lacking one or both eyes. Twelve of the 25 had hare-lips and cleft palates. Nearly all the severely deformed foetuses had receding lower jaws. Eighteen of the monstrosities had deformed feet; some lacked digits; in others digits were fused so that the developing paws were forked. Several of the most severely distorted embryos, however, had well-formed feet and toes which were the only recognizable external features.

These results showed that freezing hamsters for 30 min. and for 45 min. had entirely different effects during the first half and identical effects during the second half of pregnancy as judged by subsequent foetal development. This suggested that the amount of ice formed in hamsters partially frozen during the first half of pregnancy might determine whether normal foetal development continued. Foetal resorption had occurred in animals which became supercooled at 9½, 10½, and 11½ days of gestation which indicated that this critical period in the second half of pregnancy did not depend upon freezing (Smith, 1956*c*).

The proportion of body-water frozen

The percentage of water converted to ice in partially frozen pregnant hamsters was calculated by Lovelock's method (see Lovelock & Smith, 1956). It varied from 9 to 27 per cent. and averaged 19 per cent. in hamsters which had been freezing for 30 min. The range was from 16 to 49·6 per cent. and the average was 30 per cent. in those frozen for 45 min.

Two hamsters which had been pregnant for $4\frac{1}{2}$ days were frozen for 30 min. and for 45 min. respectively. Their abdomens were then opened. The uterus felt soft in the animal frozen for 30 min. but was partially coated by thin plaques of ice and felt firm in the animal frozen for 45 min.

The effect of cooling to body temperatures between 15° and 20° C. at $10\frac{1}{2}$ days of pregnancy

Two hamsters which had been pregnant for $10\frac{1}{2}$ days were cooled in the usual way in closed vessels at $+2°$ C. until they became comatose. Their colonic temperatures were 15° and 17° C. They were left to re-warm at room temperature and within 40 min. had turned over and within 2 hours had apparently recovered. Small quantities of blood oozed from the vagina of both animals during the next 2 days. They were killed at $13\frac{1}{2}$ days of pregnancy. Nine resorbing foetuses were found in one animal and 5 in the other. The foetal sacs contained blood-stained fluid and clots. The appearances were indistinguishable from those found in hamsters which had been frozen for 30 min. or for 45 min. at $10\frac{1}{2}$ days and killed at $13\frac{1}{2}$ days of pregnancy.

Hamsters which had been pregnant $9\frac{1}{2}$ and $11\frac{1}{2}$ days were also allowed to re-warm from body temperatures between 15° and 17° C. and killed at $13\frac{1}{2}$ days. The majority of foetuses were resorbing although three normal foetuses were also present in the litter from an animal cooled at $11\frac{1}{2}$ days.

DISCUSSION

It was remarkable that pregnant hamsters survived respiratory and cardiac arrest followed by freezing for 30 min. and that foetal development continued normally in animals so treated at $12\frac{1}{2}$ days and between $1\frac{1}{2}$ and $8\frac{1}{2}$ days of gestation. The uterine haemorrhage and foetal resorption which took place in hamsters cooled at $9\frac{1}{2}$, $10\frac{1}{2}$, and $11\frac{1}{2}$ days of pregnancy clearly did not depend on arrest of circulation and respiration or on freezing because it occurred in animals allowed to re-warm from body temperatures of 15 to 17° C. It was probably analogous to the haemorrhage observed by Courrier & Marois (1953) in pregnant rats cooled to between 16·5 and 20° C. on or after the 12th day when, because of the longer gestation period, foetal and placental development would have reached about the same stage as in the hamster at $9\frac{1}{2}$ days. Similarly, the prolonged parturition, stillbirths, and deaths among hamsters frozen on the 13th to 15th days of pregnancy may not have been the result of freezing. They were probably caused in the same way as identical effects observed by Vidovic (1956)

in rats cooled during the last 3 days of gestation to body temperatures between 17° and 20° C. Giaja's closed vessel technique (Giaja & Andjus, 1949), which had been used by Courrier & Marois and by Vidovic for cooling pregnant rats, was used in the first stage of cooling the hamsters. The question arises whether the reduced body temperature was primarily responsible for the haemorrhage and foetal deaths produced at critical stages of gestation in these animals or whether either the raised carbon dioxide or the diminished oxygen tension caused the damage. The source of bleeding might have been the embryo itself or the embryonic membranes and placenta. It may be significant that the hamster placenta undergoes rapid growth and a radical change in structure between the 9th and 11th days of pregnancy. During this period, when cooling by the technique used has such disastrous results, the allantois reaches the chorion and fuses with it to form a haemochorial placenta. The rapidly growing blood-vessels might be particularly vulnerable to damage. Between 12 and $12\frac{1}{2}$ days further changes take place and the placenta becomes haemoendothelial in type (Boyer, 1948; Adams & Hillemann, 1956). The effects on the placenta of cooling and freezing hamsters are now being studied in collaboration with Professor Amoroso.

The most interesting of all the results obtained in these experiments were the abnormal foetuses found in animals frozen for 45 min. during the first $8\frac{1}{2}$ days of pregnancy. It seems unlikely that they were due to placental damage because the blastocyst does not become embedded until $4\frac{1}{2}$ days and a placental cone only begins to appear on the 7th day. The question arises whether oxygen lack could be responsible. Reduced oxygen tension is well tolerated by new-born mammals and by foetuses in advanced stages of development (Himwich *et al.*, 1941, 1942; Hicks, 1953) but may induce foetal abnormalities in mice subjected to it at intermediate stages of pregnancy (Ingalls, Curley, & Prindle, 1952; Murakami, Kameyama, & Kato, 1956). The hamsters were exposed to severe oxygen lack during the first stage of cooling and again during the second stage of cooling after cessation of breathing and circulation. Oxygen requirements must, on the other hand, have been greatly reduced when the temperature throughout the body fell below zero and when body-water was crystallizing as ice. It seemed unlikely that the foetal abnormalities produced by freezing for 45 min. but not by freezing for 30 min. could have been due to an extra 15 min. of hypoxia. The increase from 20 per cent. to 30 per cent. in the proportion of body-water frozen and the accompanying increase in concentration of solutes in the medium surrounding the embryos might, on the other hand, have been decisive.

Anencephaly, pseudencephaly, hydrocephalus, spina bifida, defects of the eyes, hare-lip, cleft palate, and deformities of the feet have been produced in mammalian foetuses by treating the mother with nitrogen mustards, trypan blue, phenyl mercuric acetate, aminopteroyl glutamic acid, and other poisons and folic acid antagonists (Hicks, 1954; Gillman, Gilbert, & Gillman, 1948; Nelson, Asling, & Evans, 1952; Thiersch, 1952). Prolonged deficiency of vitamins in the

diet before and during pregnancy may have similar effects (Giroud, 1954; Warkany, 1954). It seems improbable that freezing, which produced identical foetal abnormalities, could have acted in the same way as any of these toxic substances or that it could have imitated a dietary deficiency. The same malformations are produced by X-irradiating pregnant rodents at critical periods just before and during organogenesis (Russell, 1950; Russell & Russell, 1954 *a*, *b*). X-rays produce ionization and are thought to affect embryonic development by damaging the nuclei, particularly in dividing cells. Chromosome aberrations, which vary according to the stage of mitosis, are produced. It is possible that freezing might produce some of these actions. Prolonged or intense cold is known to affect dividing plant cells and may cause polyploidy. It might well have analogous effects in mammalian embryos, perhaps by increasing intracellular viscosity and causing chromosomes to stick together during mitosis. Irradiation of mouse embryos before implantation leads to a very low incidence of abnormalities. The suggestion is that irradiation at early stages killed some blastomeres but that the remainder, which were totipotent continued normal development (Russell & Russell, 1954 *a*, *b*). On the other hand, hamster embryos frozen at $2\frac{1}{2}$ days when they were morulae developed as monstrosities. The question arises whether the cells composing the hamster morula have already become differentiated, so that death of a few cells leads to abnormal development, or whether freezing modifies the potentialities of some or all the component cells. Much work remains to be done, but there can be little doubt that the use of intense cold will illuminate a number of problems in mammalian embryology.

SUMMARY

1. Golden hamsters at different stages of pregnancy were cooled until breathing and heart-beats stopped and until they froze with deep body temperatures below 0° C. They were subsequently resuscitated.

2. Animals frozen for 30 min. at $1\frac{1}{2}$–$8\frac{1}{2}$ days or at $12\frac{1}{2}$ days had normal numbers of well-developed foetuses when killed at $13\frac{1}{2}$ days of gestation. Others frozen for 30 min. at these times gave birth to litters and reared young.

3. Freezing for 45 min. at $12\frac{1}{2}$ days of gestation was also harmless as judged by the number and appearance of foetuses at $13\frac{1}{2}$ days.

4. By contrast, freezing for 45 min. at $1\frac{1}{2}$–$8\frac{1}{2}$ days of gestation arrested development or caused malformation of most of the foetuses. The abnormalities included hydrocephalus, anencephaly, herniation of the brain, absence of one or both eyes, hare-lip, cleft palate, and deformities of the feet.

5. There was a critical period of pregnancy between $9\frac{1}{2}$ and $11\frac{1}{2}$ days when uterine haemorrhage and foetal resorption resulted whether the hamsters were allowed to re-warm from body temperatures of 15–17 C. or whether they had been chilled below zero and frozen for 30–45 min.

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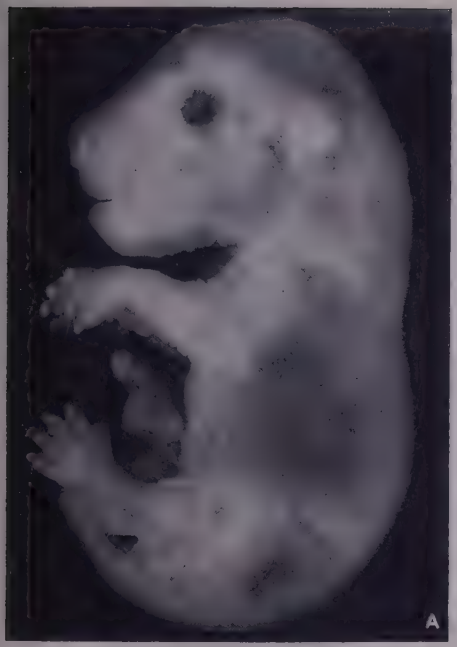
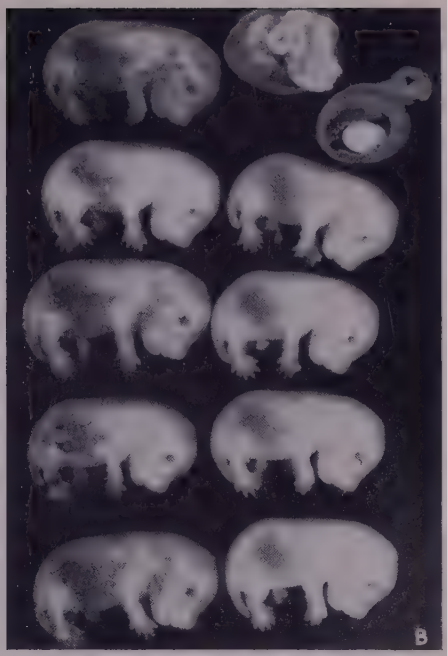
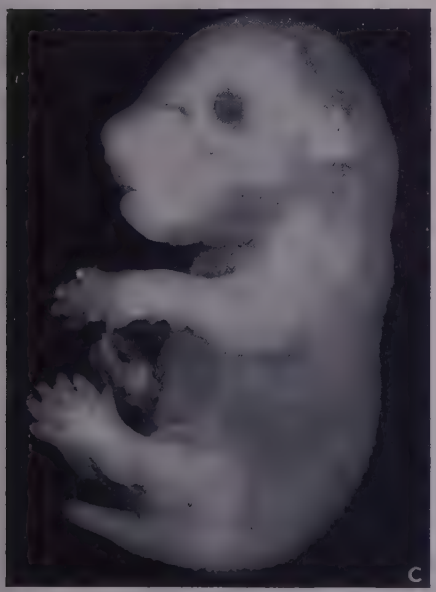
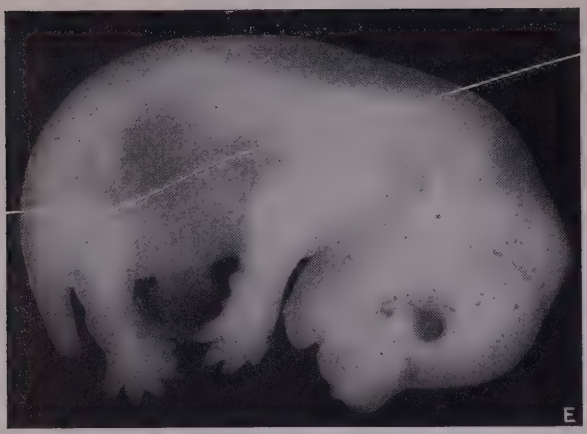


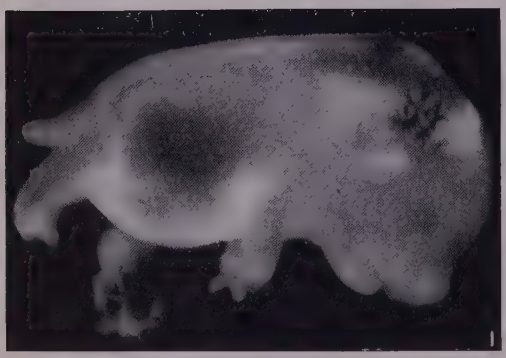




A. U. SMITH

Plate 1





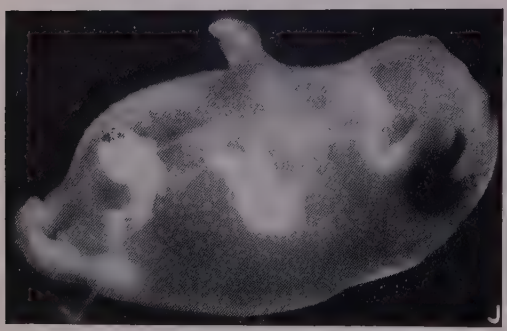


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Plate 2

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EXPLANATION OF PLATES

Plate 1

Fig. A. Normal 13½-day foetus. ×5.

Fig. B. Litter of 13½-day foetuses from a hamster frozen for 30 min. at 3½ days of gestation. ×1·2.

Fig. C. The only foetus at 13½ days of gestation from a hamster frozen for 30 min. at 9½ days. ×5.

Fig. D. Litter of resorbing foetuses found at 13½ days of gestation in a hamster frozen for 30 min. at 10½ days. ×2·3.

Plate 2

Fig. E. An undersized foetus found at 13½ days of gestation in a hamster frozen for 30 min. at 11½ days. ×6·7.

Figs. F, G, H. Abnormal foetuses found at 13½ days of gestation in a hamster frozen for 45 min. at 2½ days. ×2·3.

Figs. I, J. Abnormal foetuses found at 13½ days of gestation in a hamster frozen for 45 min. at 6½ days. ×5.

Fig. K. Litter of abnormal or resorbing foetuses found at 13½ days of gestation in a hamster frozen for 45 min. at 7½ days of pregnancy. ×1·2.

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Metabolic Characteristics of the Heart-forming Areas of the Early Chick Embryo

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WITH THREE PLATES

INTRODUCTION

OUR knowledge of the sequence of events that culminate in the onset of contractility in the heart of the early chick embryo has been evaluated by Ebert, Tolman, Mun, & Albright (1955). Immunochemical analyses made during the initial phases of cardiogenesis, which precede the appearance of recognizable cardiac primordia, indicate that in the embryo at the head-process stage the distribution of the proteins, cardiac myosin (Ebert, 1953), and cardiac actin (Ebert *et al.*, 1955), coincides with the heart-forming areas as defined by isolation methods (Rawles, 1943). In earlier stages detectable quantities of cardiac actin are absent, and cardiac myosin is distributed throughout the epiblast in the embryo at the definitive primitive streak stage. Present concepts of the synthesis and distribution of the cardiac contractile proteins are based on the sensitivity of the immunochemical methods.

The progress of the research makes it appear likely that the extension of these studies through the application of precise quantitative techniques may lead to an understanding of the mechanisms underlying the loss of myosin from regions of the embryo adjacent to the heart-forming areas; in addition, however, the need to continue to search for patterns of intermediary metabolic differentiation underlying the initial phases of heart development has been emphasized. The provocative observation by Spratt (1950) that critical concentrations of sodium fluoride specifically inhibit the formation of the heart suggested that this inhibitor might be used to determine in part the degree of metabolic differentiation in the chick embryo during the initial phases of cardiogenesis and might provide

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a chemical tool for the localization of the heart-forming areas of the blastoderm.

These expectations have been realized. The objective of the present account is to present the experimental findings which demonstrate that the heart-forming regions of the early embryo are differentiated in the metabolic sense as early as the definitive primitive streak stage. A consideration of the mechanism of action of sodium fluoride in inhibiting cardiogenesis led to an analysis of the electron transport systems of the heart-forming cells. Antimycin A, an antibiotic produced by a species of streptomyces (Dunshee, Leben, Keitt, & Strong, 1949), and shown by Potter & Reif (1952) to inhibit Slater's Factor, a component of the respiratory chain, has proved to be a useful tool. As will be made clear, this inhibitor, when applied to the chick embryo cultured *in vitro*, inhibits the developing heart and mesoderm.

These findings were presented at the Annual Meeting of the American Association of Anatomists during a programme honouring Professor B. H. Willier. An abstract has been published (Ebert & Duffey, 1956).

METHODS

Embryos were obtained from eggs of New Hampshire Red stock and were prepared for explantation according to the technique of Fell & Robison (1929) as modified by Spratt (1947). The basal medium described by Howard (1953) was employed; its distinctive feature is a lower concentration of sodium chloride (123 millimolar) than that found in chick Ringer's solution. Howard's observation that this medium affords a more optimal osmotic environment for circulatory and neural development in the explanted embryo than the conventional chick Ringer's solution was confirmed fully. Phenol red was incorporated into the medium to maintain a constant check on pH during the culture period.

The inhibitors[1] were added to the basal medium and dispersed uniformly by gentle agitation of the container. The desired amounts of sodium fluoride were weighed on an analytical balance and incorporated into the medium by rinsing the weighing bottle with the medium. Antimycin A was delivered to the medium by aliquots from a solution containing 100 μg. of the antibiotic per ml. of 95 per cent. ethyl alcohol. Embryos were explanted immediately after solidification of the medium. At recovery, the embryos were fixed in Kleinenberg's picro-sulphuric solution and stained with Conklin's hematoxylin.

RESULTS

1. *Inhibition by sodium fluoride*

The actions of sodium fluoride on embryos of stages 3$^+$ through 8$^-$, according to the Hamburger & Hamilton (1951) series of normal stages, are directed

[1] Chemicals used in the study were purchased from the following sources: Antimycin A, Wisconsin Alumni Research Foundation; sodium fluoride, Coleman and Bell; sodium pyruvate, Nutritional Biochemicals Corporation; alpha tocopherol, General Biochemicals, Inc.

primarily at the prospective heart-forming areas. In concentrations ranging from 5×10^{-3} M to 2×10^{-2} M, during culture periods of 2 to 8 hours, sodium fluoride produces specific bilateral sites of degeneration in all stages explanted. After preliminary experiments in which the concentration of fluoride and the culture period were varied, it was found that the optimal conditions for specific inhibitions are 10^{-2} M sodium fluoride and a culture period of about 5 hours at 37·5° C. A total of 210 embryos has been explanted to media containing 5×10^{-3} M, 10^{-2} M, and 2×10^{-2} M sodium fluoride (Table 1). Although individual differences, such as errors in staging and variations in the amount of energy reserves transported with the embryos, may cause slight divergences in the results, the site and incidence of inhibited areas are consistent within the respective stages of embryos investigated. Inhibited areas are absent in control embryos which were cultured on medium lacking fluoride (Plate 1, figs. 1, 2, 3).

TABLE 1

Fluoride inhibition of the chick embryo cultured in vitro *for 5 hours on various concentrations of sodium fluoride*

Stage (Hamburger and Hamilton)	*3+*	*4*	*5*	*6*	*7*	*8−*
Controls (without fluoride):						
Abnormal	1	1	..	..	..	..
Normal	8	8	13	8	2	2
Total = 43						
5×10^{-3} M NaF:						
Total inhibition	..	2	..	..	..	..
Node and streak inhibition only	2	3	11	5	4	1
Inhibition of heart-forming areas	6	11	1	3	1	1
Total = 51						
10^{-2} M NaF:						
Total inhibition	24	5	4	..	..	..
Node and streak inhibition only	9	11	11	4	6	..
Inhibition of heart-forming areas	17	23	16	2	5	5
Total = 142						
2×10^{-2} M NaF:						
Total inhibition	1	4	2	3	1	..
Node and streak inhibition only	..	1	3	..	..	..
Inhibition of heart-forming areas	..	..	2	..	..	..
Total = 17						

The entire blastoderm at the 3+ stage of development is highly sensitive to fluoride. Total inhibition, which was determined by the disintegration of the embryo, occurs in about 48 per cent. of the explants cultured on 10^{-2} M fluoride. Specific sites of inhibition, the centres of which are located about 0·4 mm. anterolateral to Hensen's node, occur in about 31 per cent. of the embryos. The size and the shape of the inhibited areas are variable. In general, these areas are circular and have a diameter of 0·3 to 0·4 mm. It would be desirable to have precise dimensions of the inhibited areas which could be compared with the data

of Rawles (1943) for the heart-forming areas; however, such measurements are not meaningful because inconsistent degrees of shrinkage and wrinkling occur in the blastoderms cultured on fluoride. Nevertheless, the approximate dimensions of the inhibited areas and their locations relative to the node indicate that they lie within the prospective heart-forming areas (Plate 1, fig. 4).

A decrease in overall susceptibility to fluoride with a concomitant increase in the incidence of specific loci registering high fluoride-sensitivity is characteristic of stage 4. Bilateral sites of inhibition occur in about 59 per cent. of the stage 4 embryos. The high incidence of these fluoride-sensitive areas is maintained in all the older stages investigated. The sites of fluoride-sensitivity in stages 4 through 7 are successively located slightly anterolateral to the node, adjacent to the anterior projection of the notochord and immediately posterior to the head fold (Plate 1, figs. 5, 6; Plate 2, fig. 7).

TABLE 2

Fluoride inhibition of the chick embryo cultured in vitro *for 20 hours on medium containing* 5×10^{-3} *M sodium fluoride, and the prevention of fluoride inhibition by sodium pyruvate*

Initial stage	*4*	*5*	*6*	*7*	*8–*
Controls (without fluoride):					
Abnormal	3	0	0	0	1
Normal	10	5	7	11	20
Total = 57					
5×10^{-3} M NaF:					
Total inhibition	4	1	0	2	0
Inhibition of heart	1	1	3	3	5
Total = 20					
10^{-2} M NaF:					
Total inhibition	7	0	0	1	0
Inhibition of heart	3	2	1	5	5
Total = 24					
5×10^{-3} M NaF and 10^{-2} M Na-pyruvate:					
Inhibition absent	7	3	2	3	5
Inhibition of heart	0	0	0	0	0
Total = 20					
10^{-2} M NaF and 10^{-2} M Na-pyruvate:					
Total inhibition	5	0	1	2	1
Inhibition of heart	1	0	0	4	4
Total = 18					

By reducing the fluoride concentration to 5×10^{-3} M and increasing the culture period, the effects of fluoride on later stages have been analysed. The experiments have been performed both on intact embryos bearing a narrow ring of area opaca and on embryos separated from the posterior streak region by a transverse cut made approximately 0·8 mm. behind the node. A total of 20 embryos ranging from stages 4 to 8 have been cultured on media containing

fluoride at this concentration. Differential inhibition occurred in approximately 65 per cent. of the experimental cases (Table 2; Plate 2, figs. 8, 9, 10, 11). Fifty-one embryos in equivalent stages cultured on media lacking fluoride exhibited normal development within the limits of the *in vitro* technique (Table 2; Plate 3, figs. 14, 15).

Thus an ontogenetic series of fluoride-inhibited embryos had been obtained. The translocation of sites of fluoride sensitivity stages 3^+ to 8^- reflects the anteromedial changes in the positions of the heart-forming areas. After the morphogenesis of adjacent structures, such as the brain and fore-gut, the sites of inhibition are observed anterior to the splanchnopleuric folds of the anterior intestinal portal. In the later stages, i.e. stages 9 and 10, the cardiac elements are completely absent or retarded markedly. Retarded hearts possess essentially normal conformation, but are reduced in size, and lack the capacity to pulsate either spontaneously or after stimulation by a fine steel needle. It will be noted also (Table 2) that the inhibition by fluoride is prevented by the addition of sufficient quantities of sodium pyruvate to the basal medium (cf. Spratt, 1950).

2. *Inhibition by antimycin A*

The effects of antimycin A on the chick embryo cultured *in vitro* are illustrated in Plate 3, figs. 16, 17, and the data are summarized in Table 3. A series of embryos ranging from stages 4 through 9 have been analysed for the effects of antimycin A at concentrations of 0·01 γ to 0·50 γ per ml. of medium. Inhibitory effects occur under the following conditions: The embryos are cultured for 10 to 20 hours at 37·5° C. on approximately 2 ml. of albumen–saline–agar medium containing antimycin A. Concentrations of 0·01 to 0·02 μg. of the inhibitor per ml. of medium are virtually ineffective in inhibiting any phase of development during the culture period. Concentrations of the inhibitor above 0·05 μg. per ml. effect extensive tissue breakdown with the result that, in a 10-hour culture period, embryos in stages 4 to 7 are reduced to a structureless layer of cells and detritus. At high concentrations, a few embryos explanted at stages 8^- to 9 succeed in forming a retarded brain and a shallow fore-gut, but in such embryos the heart and somites are either completely absent or in a state of disintegration. At the optimal concentrations of 0·03 to 0·05 γ of antimycin A per ml. of medium, ectodermal and endodermal derivatives are not inhibited noticeably. Typically, the embryos cultured in this concentration for 20 hours possess a well-developed brain and spinal cord flanked by large bulging vesicular areas which extend the length of the embryonic axial structures and toward the area opaca vasculosa. Within these vesicular spaces, overlain with ectoderm and floored with endoderm, mesodermal tissues may be observed in advanced stages of lysis and fragmentation. Included in these mesodermal tissues which are susceptible to antimycin A are the heart and lateral mesoderm derivatives, mesoderm of the head region, and somites. It is important to emphasize that somite formation is not prevented; shortly after the epigenesis of the general somite

pattern, lysis of the somites occurs. In the presence of the inhibitor, the segmental plate mesoderm remains capable of somite differentiation, but the somites formed are not maintained. Such a sequence of events may occur in the head mesoderm and the undifferentiated lateral reaches of the mesoderm as well, but, since no well-defined structures normally arise from these tissues during the phases of development investigated, no morphological criteria are available for critically analysing the effects of the inhibitor.

TABLE 3

Inhibition of heart formation and function, somite development, and general development in the chick embryo cultured in vitro *on medium containing 0·05 γ antimycin A per ml.*

	Initial stage	*No. of cases*	*Average stage after 20 hours' culture**	*Average no. somites embryo*	*Per cent. heart formation*	*Per cent. heart beat*
Controls	4	14	10	9·6	93	50
	5	5	10	12	100	100
	6	5	11	12	100	100
	7	11	11	13	100	100
	8⁻	7	12	15	100	86
	8, 9	6	12	16	100	100
Antimycin A	4	6	6	1	0	0
	5	4	7	2	0	0
	6	5	7	1	0	0
	7	8	8	2	0	0
	8⁻	3	11	6	0	0
	8, 9	4	11	3	0	0

* The embryos were staged at the end of the culture period according to the normal stages of Hamburger & Hamilton (1951). The characteristics of neural development were the criteria used in staging inhibited embryos.

A syndrome of abnormalities similar in several respects to those we have described results from treatment of the chick embryo with the purine antagonist 8-Azaguanine. Two groups of investigators, Waddington, Feldman, & Perry (1955) and Frair & Woodside (1956), have emphasized that 8-Azaguanine produces a degeneration of already formed somites.

The heart is highly susceptible to antimycin A. Indeed, the heart appears to be the most susceptible mesodermal structure. At concentrations of 0·03 to 0·05 γ antimycin A per ml. of medium, the embryonic heart either is completely absent or is represented by paired primordia in retarded stages of fusion, the most advanced of which are unable to pulsate even when stimulated by a needle prick.

The effects of antimycin A on the definitive primitive streak blastoderm (stage 4) differ significantly from those produced by sodium fluoride. Whereas fluoride elicits localized areas of inhibition in the early stages, antimycin A fails

to produce localized sites of inhibition which can be reproduced consistently. The antibiotic has produced vesicular areas in a few cases; however, these vesicles usually arise first alongside the streak and around the node and then, with time, enlarge to the limits of the area pellucida. The production of such vesicular areas is nearly always followed by a complete breakdown of the entire blastoderm. This may be contrasted with the production of large vesicular areas predominantly in the lateral mesoderm of stages 6, 7, and 8^-, as described above. From between the latter vesicles, the embryonic neural tube arises and continues to develop into a normally proportioned brain and spinal cord.

3. *Attempts to prevent antimycin A inhibition*

The question arises next as to the mechanism of action of antimycin A in inhibiting the mesodermal components of the explanted embryo. Nason & Lehman (1955, 1956), using a 10- to 25-fold purified particulate fraction from rat skeletal muscle, have demonstrated convincingly that *d*-alpha-tocopherol exerts a marked enhancing effect on the rate of DPNH oxidation, specifically on the reduction of cytochrome *c*. Isooctane extraction of the particulate fraction resulted in a 75 to 95 per cent. decrease in cytochrome *c* reductase activity, although only 10 per cent. of the enzyme lipid was removed. Enzymatic activity was restored completely upon the addition of the tocopherols or *d*-alpha-tocopheroxide. The residue resulting from vacuum distillation of the isooctane used in the extraction procedure also can restore the enzymatic activity. This residue does not contain free tocopherol, but it is somewhat more active than tocopherol on a weight basis. The particulate DPN-cytochrome *c* reductase is inhibited by antimycin A, a reaction which is reversed by tocopherol. Therefore, it appeared essential to determine whether the antimycin A inhibition described in the early embryo could be prevented by tocopherol.

Alpha-tocopherol at concentrations ranging from 10 to 200 γ per ml. of medium was tested for its possible ability to block the activity of 0·05 γ per ml. antimycin A. The tocopherol was homogenized with the albumen portion of the medium in a Ten-Broeck homogenizer in order to successfully incorporate the vitamin into the medium (modification of the technique of Ames & Risley, 1949). Analyses have been made on sixty explants cultured according to the conventional techniques. In the albumen concentrations routinely employed (Spratt, 1947), prevention of the antimycin A effect has not been obtained with α-tocopherol at any of the embryonic stages utilized. In contrast, we have found that by increasing the albumen content of the medium from 15 to 30 per cent. the effect of 0·05 γ antimycin A per ml. of medium is completely removed. These results are consistent with the argument advanced by Nason & Lehman (1956) and Nason, Averbach, & Terrel (1956) that the extracted tocopherol-like component from the particulate fraction of muscle is not free tocopherol and that a lipid bound to beef serum albumin can be substituted for tocopherol in the restoration of activity in the DPN- and succinate-cytochrome *c* reductases. How-

ever, these investigators reported that crystalline egg albumin and human γ-globulin, relatively free of lipids, fail to restore activity in these systems. It is likely that a component similar to the lipid of beef serum albumin is present in low concentrations in egg albumen. Such a possibility is not remote in view of the fact that Nason, Averbach, & Terrel have suggested that the lipid attached to beef serum albumin and the isooctane extractable component from particulates may have a common identity.

DISCUSSION

A series of embryos covering the period from the intermediate streak stage to the onset of contractility of the heart, formation of the foregut, formation of the primary divisions of the brain, advanced somite formation, and elaboration of functional extra-embryonic and embryonic circulatory systems has been analysed for the effects of the inhibitors sodium fluoride and antimycin A on the development of the heart and other mesodermal elements. The demonstration that the heart-forming areas are differentiated metabolically as early as the definitive primitive streak stage poses a series of further questions for analysis. One may ask, first, whether other lines of evidence justify the application of the term 'heart-forming areas' to the regions affected by fluoride. It is important to note that embryos may be cultured for brief intervals in a medium containing fluoride, the initial sites of inhibition observed and marked with fine carbon particles, and the embryo transferred to the basal medium lacking the inhibitor. These embryos recover and undergo relatively normal development. The number of cases is insufficient for detailed analysis, but it may be noted that pulsatile hearts bearing carbon marks on the ventricular wall have been recovered. The recovery of embryos exposed to 10^{-2} M sodium fluoride for as long as 30 minutes before being transferred to a medium lacking fluoride has been consistent. Of a total of 29 such embryos transferred by both authors 26 have developed pulsating hearts. However, the results of experiments using the carbon-marking technique have been less reproducible, for the method requires the insertion of a particle of blood charcoal into the floor of one of the vesicular sites of degeneration. A comparison of these findings with the results of experiments in which carbon particles are inserted between the epiblast and hypoblast of normal embryos must be completed before an evaluation is made. The findings will be published subsequently.

Demonstration of the fluoride-sensitive heart-forming areas has been done also *in ovo* after injection of sodium fluoride into the yolk; however, the results have been inconsistent, probably because of unequal distribution of the inhibitor in the yolk. Embryos inhibited *in ovo* have been explanted to basal medium and have exhibited complete recovery during the culture period. The coupling of an inhibition phase with a recovery phase presents an opportunity for the isolation of cells from the well demarcated cardiogenic centre.

The correlation of results from the studies using fluoride with those from immunochemical analyses and studies of the potency of chorio-allantoic grafts of fragments of blastoderms present some striking relationships. The capacity of the blastoderm to form heart-tissue is gradually restricted from its widespread distribution in pre-streak stages to well-defined areas in the head process stage. Likewise, cardiac myosin is uniformly distributed in the epiblast in stages prior to the head process stage, but thereafter is localized to areas corresponding to the heart-forming areas. Differential areas of fluoride sensitivity also appear during the period of development between the pre-streak stage and the head process stage. The analysis of the present series of long streak, definitive streak, and head process stage embryos demonstrating fluoride sensitivity provides a sound basis for the concept that a decrease in the susceptibility of the entire blastoderm is paralleled by an increase in the incidence of defined fluoride-sensitive areas as well as by the localization of cardiogenic potency, the localization of the cardiac myosin-synthesizing system, and the appearance of a cardiac actin-synthesizing system. Although studies have not been made to determine whether the synthesis of cardiac myosin and actin is affected in the heart-forming areas, one can hardly conceive, after examination of the inhibited areas, that synthesis and breakdown of these proteins is not affected. According to Needham (1932), protein catabolism terminating in ammonia production attends fluoride inhibition of anaerobic glycolysis in the chick embryo. The accumulation of such products may account for the edematous and vesicular appearance of the fluoride-sensitive areas.

In a comparison of the effects of sodium fluoride and antimycin A, several points of difference emerge. First, differential inhibition due to the latter inhibitor could not be demonstrated prior to the time of head fold formation. In the stages examined, the antimycin A-sensitive systems characterize the mesoderm, as contrasted with the insensitive ectoderm and endoderm, but are not segregated to specific mesodermal regions. Second, the sequence of events in the effect of antimycin A on the somites may be reiterated: the formation of the somites is not affected; the somites form, but do not persist. Somites have not yet been sub-cultured to a basal medium after exposure to the inhibitor; such an experiment has obvious interest.

Spratt (1948, 1950) reported the differential susceptibility of the brain and heart in chick embryos cultured *in vitro* to oxygen deficiency, cyanide, and iodoacetate, and to fluoride respectively. On the basis of his findings, he concluded that, at the concentrations of these inhibitors (cyanide, 10^{-2} M; iodoacetate, 2×10^{-5} M; fluoride, 5×10^{-3} M) which produce differential effects, brain is more sensitive to conditions which retard enzymes that catalyse respiration and heart is more sensitive to those inhibitors which act on glycolysing enzymes.

The resistance of the heart of the embryo, fetus, or new-born to anaerobiosis has been known for many years. Burrows (1921) found that fragments of 4- to

5-day embryonic chick heart cultured on plasma clots in 'pure' nitrogen are capable of active growth, cellular division, and pulsation for over 40 hours; however, heart-tissue from 10- to 15-day embryos under the same conditions fails to function or survive. Wind (1926) confirmed these differences in susceptibility to anaerobiosis in young and old hearts; however, the successful repetition of Burrows's finding was made in an atmosphere of 2×10^{-4} volume per cent. of oxygen. In the adult rat, De Haan (1956) demonstrated that electrical activity in the ventricles of the heart continues for about 5 minutes when the animal is subjected to total anoxia. In the 1-day-old rat under similar circumstances, the ventricular ECG can be recorded for a mean period of about 31 minutes. Prolonged exposure to anaerobic conditions is fatal for the heart at any age. The observed resistance of the heart of the embryo or new-born has not been explained. De Haan (1956) has put forward the hypothesis that one source of irreversible change occurring in tissues exposed to an anoxic environment might be protein breakdown by cathepsins activated in an acid medium; this acidity being supplied by excessive lactic acid production arising from anaerobic metabolism. His findings with adult heart are in consonance with this argument. Although De Haan's studies of lactic acid production and proteolytic activity in the day-old animal support this idea, no information has been advanced concerning either the susceptibility of infant versus adult protein to the action of the proteolytic enzymes or differences in sarcoplasmic organization in the heart at the stages examined.

The course of respiration in the chick heart has been found to decrease during days 4 to 6 and then to decrease further after day 7 (composite trend indicated by the works of Warburg & Kubowitz, 1927; Hughes, 1949). Sippel (1954) has correlated this decrease in oxygen consumption with the trend in succinoxidase activity of the heart during the entire incubation period, the first 18 hours excepted. Succinoxidase activity increases rapidly during the first 48 hours and then levels off. A second increase in activity commences at about the 9th day and continues gradually through hatching. Sippel has suggested that mitochondrial increments and differentiations attendant with reorganization of these elements in relation to the myofibrils may result in increased efficiency of oxygen utilization by the succinoxidase system and in increased efficiency of utilization of the energy produced.

The inconsistencies in the reported effects of 10^{-4} M and 2×10^{-5} M moniodoacetate and 2×10^{-2} M, 10^{-2} M, and 5×10^{-3} M sodium fluoride should be considered against this background. Both iodoacetate and fluoride are strong inhibitors of glycolysis. It can be argued that, at the higher levels of concentration of the two inhibitors, the glycolytic mechanisms of both brain and heart are completely blocked. However, at the lower concentrations, differential inhibition occurs. Spratt advanced the hypothesis that this difference between iodoacetate and fluoride at lower concentrations is attributable to brain being primarily dependent on (aerobic) respiration, which Fuhrman & Field (1943)

have shown is inhibited by iodoacetate, whereas, because of its inhibition by fluoride, heart is thought to depend on glycolysis. The improbability of this hypothesis stems from the misinterpretation of the results of Fuhrman & Field as well as the failure to utilize information pertaining to fluoride inhibition of intracellular oxidation. The work of Fuhrman & Field on anaerobic glycolysis and respiration in tissue slices of rat cerebral cortex in the presence of iodoacetate has been summarized by them in the statement that their data are in harmony with the view of Turner that '. . . in comparable samples (of carrot tissue) iodoacetate acts more rapidly in depressing the rate of fermentation than it does in depressing the rate of oxygen uptake associated with true carbohydrate respiration'. Similar conditions have been found by Needham (1932) in the iodoacetate poisoning of the chick embryo. Likewise, Needham found that, at concentrations of fluoride which are sufficient to abolish anaerobic glycolysis, respiration is not affected; however, increased fluoride concentrations depress respiration. (The latent period between the onset of the inhibition of glycolysis and depression of respiration may be explained by the presence of glycolytic intermediates which continue to act as substrates for various respiratory processes after glycolysis is blocked.)

Iodoacetate and fluoride inhibit a number of enzymes in addition to those catalysing glycolysis (Sumner & Somers, 1953). The discrepancies between the experimental results and the picture of inhibition which one might suspect in iodoacetate and fluoride inhibition can probably be explained in part by the high degree of unspecificity of action of these two substances. Spratt (1950) was led to conclude that enolase is the primary site of fluoride action, since the inhibition is prevented by added pyruvate (cf. Table 2, Plate 2, fig. 12, and Plate 3, fig. 13). In addition to enolase inhibition, fluoride is recognized as a potent inhibitor of non-specific phosphatases and mitochondrial adenosine triphosphatase (Judah & Williams-Ashman, 1951; Potter, Le Page, & Klug, 1948). Slater & Bonner (1952) have found succinic dehydrogenase to be the site of fluoride inhibition in the succinoxidase system of heart-muscle preparations. The range of concentrations of NaF within which succinic dehydrogenase and chick heart are markedly inhibited is 2×10^{-3} M to $1{\cdot}6 \times 10^{-2}$ M. Mitochondrial ATPase activity is almost eliminated at the same magnitude of concentration. This considerable body of evidence for the fluoride inhibition of enzymes associated primarily with cytoplasmic particulates led Harman & Feigelson (1952) to investigate the effects of fluoride on mitochondrial structure. They found that fluoride protects the mitochondrial form ('target' form) which supports higher levels of oxidation and phosphorylation. It will be of interest to compare the properties of mitochondria in embryonic brain and heart; immunochemical studies of the differentiation of organ- and species-specific properties of mitochondria, which have been initiated in our laboratory, suggest that the technical problems involved are not insurmountable (Albright, 1956).

The inhibition of cellular respiration, in particular the succinic oxidase system,

by antimycin A has been demonstrated by Ahmad, Schneider, & Strong (1950). Potter & Reif (1952) reported that this antibiotic inhibits respiration in systems catalysing the oxidation of reduced diphosphopyridine nucleotide (DPN); however, an antimycin-insensitive pathway for DPN oxidation was found also. Subsequently, Reif & Potter (1954) investigated the mechanism of action of antimycin A more fully and found that the degree of inhibition of systems requiring DPN varied with the tissues used: antimycin A inhibited the oxygen consumption in DPN-coupled oxidation of malate at the rate of 81, 33, 17, and 3 per cent. in heart, kidney, liver, and Flexner-Jobling carcinoma respectively, and of D-glyceraldehyde-3-phosphate by 98, 18, 31, and 33 per cent. in the same tissues. The results suggest that alternate pathways exist in different tissues for the oxidation of DPN.

The marked sensitivity of embryonic heart and the lack of sensitivity of embryonic brain to antimcyin A should be contrasted with the observation by Spratt (1950) on the cyanide-sensitivity of these two tissues. The developing brain is cyanide-sensitive; the heart is not. Therefore, the transfer of electrons in key energy-yielding reactions in brain probably is mediated by cytochrome oxidase (since cyanide inhibits cytochrome oxidase; Keilin & Hartree, 1939), whereas the electron-transfer system of heart does not require this enzyme. Cytochrome oxidase is not absent, necessarily, but an alternate pathway must exist. In fact, cytochrome oxidase (indophenol oxidase) has been detected in chick blastoderms as early as the intermediate streak stage by use of the explanting procedure on an agar medium containing dimethyl-*p*-phenylenediamine-α naphthol (Fraser, 1956). Moreover, Fraser has stated that the development of the ability to oxidize the Nadi reagent is not autonomous in the epiblast, but that the prospective mesoderm must undergo involution before activity of the enzyme can be detected. Potter & Reif (1952) have shown clearly that antimycin A does not inhibit dehydrogenases involved in anaerobic glycolysis; hence antimycin A inhibition of heart cannot be interpreted to mean that the developing heart derives its energy from glycolytic mechanisms alone. Ahmad, Schneider, & Strong (1950), Potter & Reif (1952), and Chance (1952) have expressed the view that antimycin A inhibits the succinic oxidase system at an intermediate stage, probably the 2,3-dimercaptopropanol (BAL)-sensitive factor (Slater's Factor; Slater, 1948). It was also observed that antimycin A has no effect, even at very high concentrations, on cytochrome oxidase (Ahmad *et al.*, 1950). Thus a high degree of specificity of action has been shown for antimycin A. Though the exact site and mode of action of the antibiotic have not yet been determined, Thorn (1956) has presented evidence which suggests that the antimycin- and BAL-sensitive factors are not identical; however, they might represent different reactive groups on the same substance. The differences in the antimycin and cyanide sensitivities of chick brain and other mesodermal elements are interpreted as due to fundamental differences in the electron-transfer systems of these tissues: brain is dependent on cytochrome oxidase, but either lacks or is able to

by-pass the antimycin-sensitive factor, whereas heart is strongly dependent upon the latter intermediate, but does not require an active cytochrome oxidase. In the absence of critical information on the concentrations of iodoacetate and fluoride necessary to inhibit glycolysis and aerobic respiration in the parts of the early chick blastoderm, in view of the results of Fuhrman & Field and Needham on the actions of these inhibitors, and as a result of the pronounced effect of antimycin A on the heart and mesoderm in general, the concept that brain depends primarily on oxidative metabolism whereas heart depends on glycolysis must be reconsidered.

SUMMARY

1. The inhibition of early development by sodium fluoride and antimycin A has been analysed in the early chick embryo cultured *in vitro*.

2. The relatively specific inhibition of the development of the heart by sodium fluoride, originally reported by Spratt (1950), has been extended to include early streak stages, head process stage, and head fold stage embryos in order to determine the period during development at which the heart-forming tissues become fluoride-sensitive.

(*a*) Fluoride-sensitive areas corresponding to the bilateral prospective heart-forming areas arise in the early streak stages.

(*b*) The locations of fluoride-sensitive loci in subsequent stages mirror the sites of highest cardiogenic potency and the areas of highest levels of the cardiac proteins, myosin and actin.

3. In very low concentrations, antimycin A has a striking inhibitory action on the mesoderm of the chick embryo grown *in vitro*. The brain is free of gross developmental abnormalities in embryos in which all mesodermal derivatives, including heart, have degenerated.

4. The action of antimycin A cannot be prevented by the addition of *d*-α-tocopherol; however, the effects of this inhibitor can be prevented by doubling the albumen content of the medium. It is suggested, in consonance with recent findings by Nason *et al.*, that a lipid bound to albumin functions in the restoration of activity in the DPN- and succinate-cytochrome *c* reductases.

5. The results, together with recent information on the specificity of action of the inhibitors, provide a basis for a re-examination of the working hypothesis of Spratt (1950) which holds that brain is dependent primarily on oxidative metabolism and heart on glycolysis. The inhibition of heart by antimycin A reveals a pronounced dependence of this tissue on pathways for DPN oxidation. The combined results of Spratt and the authors, correlated with information on the action of antimycin A and other respiratory poisons, suggest that fundamental differences exist between brain and heart in their respective electron to oxygen transport systems.

ACKNOWLEDGEMENT

The authors thank Dr. J. F. Albright for his suggestion that antimycin A might prove a useful tool in the investigation.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Control embryo, explanted at stage 3+, after 5 hours' culture (4A1–40).

Fig. 2. Control embryo, explanted at stage 4, after 5 hours' culture (4A1–35).

Fig. 3. Control embryo, explanted at stage 9, after 5 hours' culture (4A1–26).

Fig. 4. Inhibition by 10^{-2} M NaF; embryo explanted at stage 3+, after 2 hours' culture (4A2–62a).

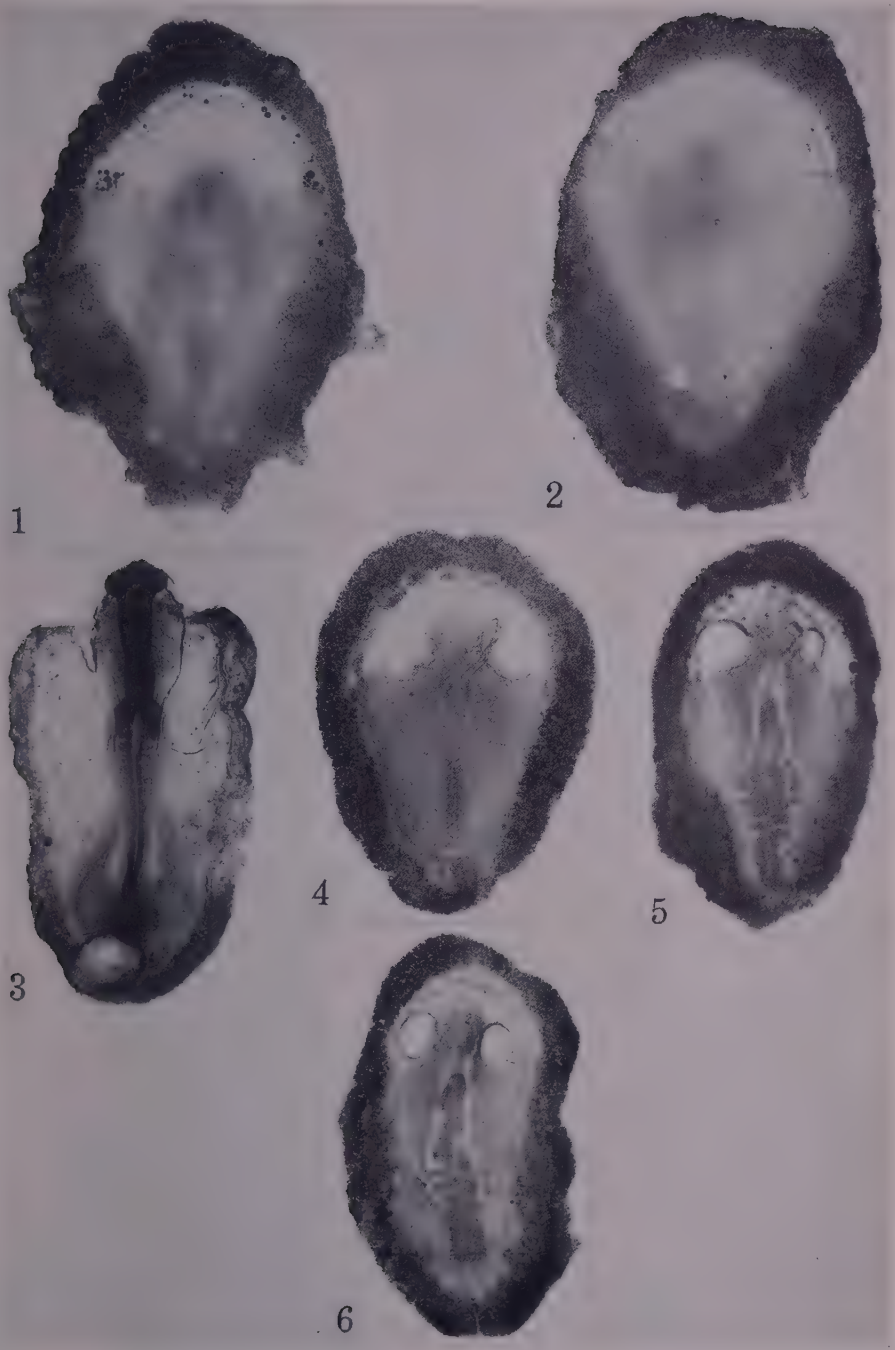
Fig. 5. Inhibition by 10^{-2} M NaF; embryo explanted at stage 5, after 4 hours' culture (4A2–95).

Fig. 6. Inhibition by 10^{-2} M NaF; embryo explanted at stage 3+, after 4 hours' culture (4A2–82).

Plate 2

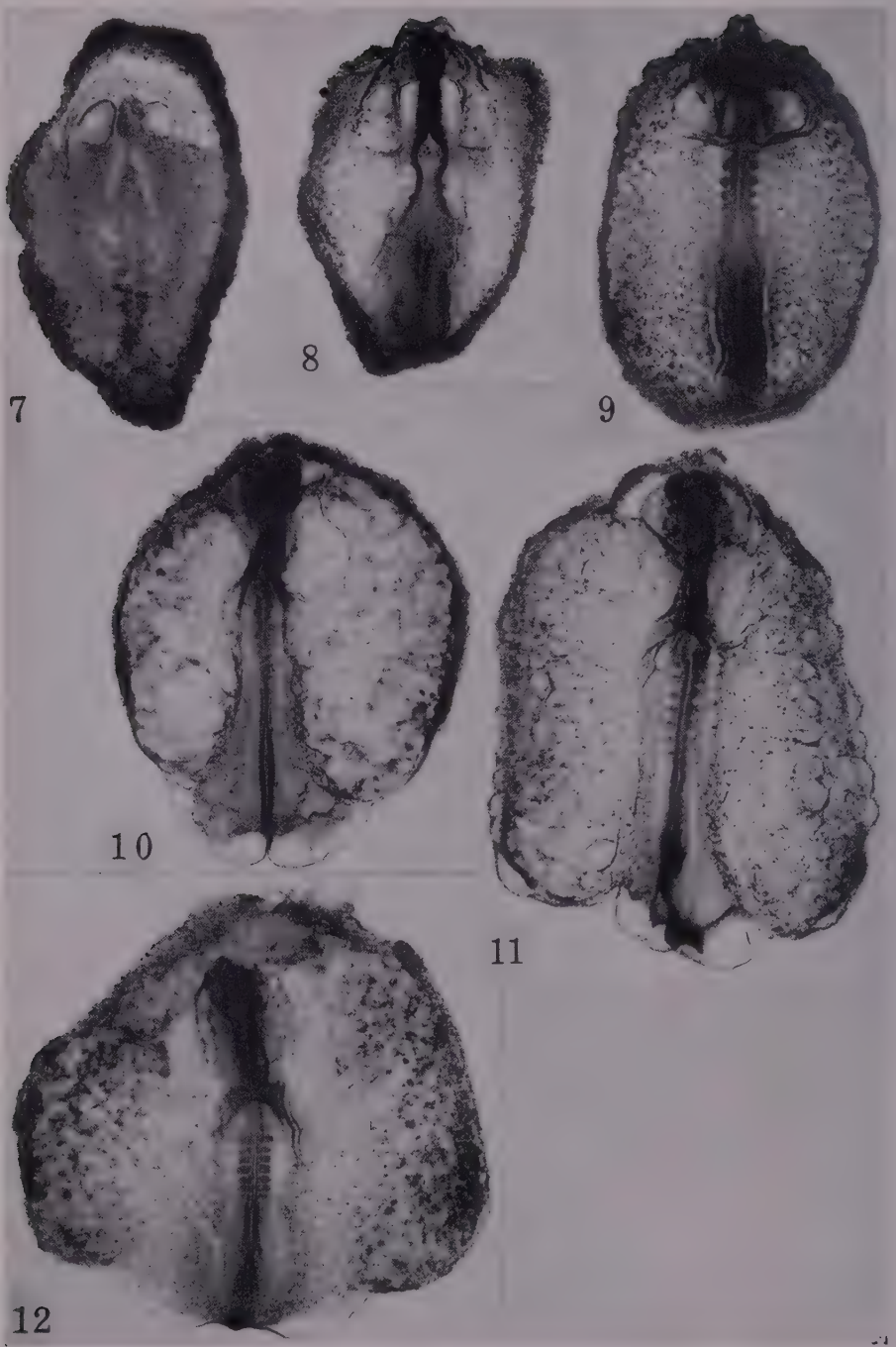
Fig. 7. Inhibition by 10^{-2} M NaF; embryo explanted at stage 4, after 4 hours' culture (4A2–83).

Fig. 8. Inhibition by 5×10^{-3} M NaF; embryo explanted at stage 6, after 5 hours' culture (4A4–46).



L. M. DUFFY *and* J. D. EBERT

Plate 1



L. M. DUFFY *and* J. D. EBERT

Plate 2

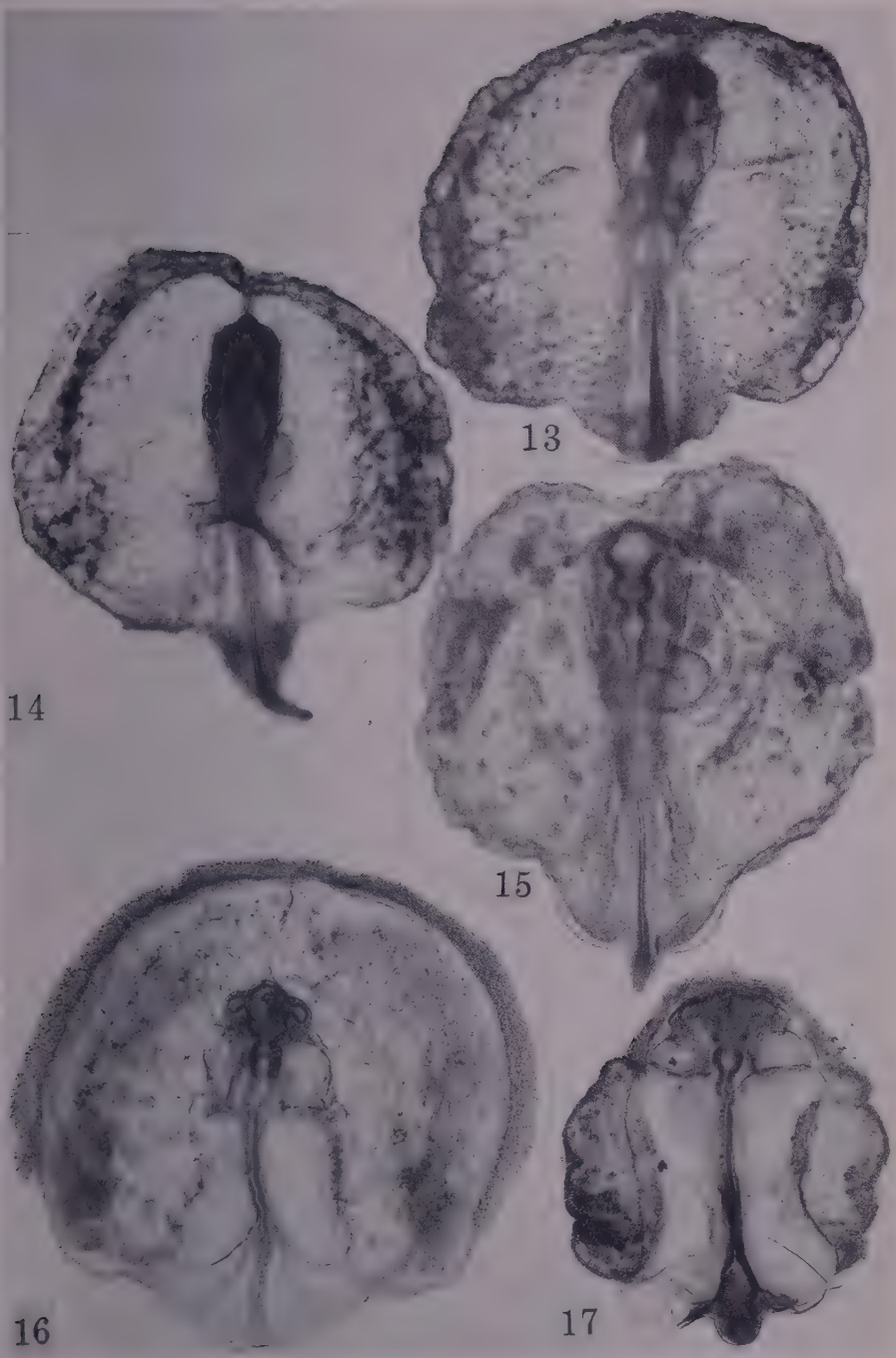


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Plate 3

FIG. 9. Inhibition by 5×10^{-3} M NaF; embryo explanted at stage 8−, after 6 hours' culture (4A4–14).

FIG. 10. Inhibition by 5×10^{-3} M NaF; embryo explanted at stage 8−, after 20 hours' culture (7A6a–9).

FIG. 11. Inhibition by 5×10^{-3} M NaF; embryo explanted at stage 8, after 20 hours' culture (7A6a–14).

FIG. 12. Reversal of the effects of 5×10^{-3} M NaF by 10^{-2} M sodium pyruvate; embryo explanted at stage 4, after 20 hours' culture (7A9a–5).

PLATE 3

FIG. 13. Reversal of the effects of 5×10^{-3} M NaF by 10^{-2} M sodium pyruvate; embryo explanted at stage 6, after 20 hours' culture (7A9a–8).

FIG. 14. Control embryo, explanted at stage 4, after 24 hours' culture (7A–6).

FIG. 15. Control embryo, explanted at stage 8−, after 24 hours' culture (7A–27).

FIG. 16. Inhibition by 0·05 γ antimycin A per ml. of medium; embryo explanted at stage 8−, after 24 hours' culture (7E–16).

FIG. 17. Inhibition by 0·05 γ antimycin A per ml. of medium; embryo explanted at stage 8, after 20 hours' culture (8R–10).

(Manuscript received 20 : xi : 56)

Studies on the Development of the Foregut in the Chick Embryo

IV. Mesodermal Induction and Mitosis

by RUTH BELLAIRS[1]

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It is generally believed that the foregut in the chick embryo is induced by the mesoderm (Waddington & Schmidt, 1933; Waddington, 1952). In particular the splanchnic mesoderm is thought to be important (Rudnick & Rawles, 1937; Rudnick, 1952). In normal development the splanchnic mesoderm becomes fused with the presumptive foregut floor whilst the gut is developing and both become considerably thickened. During this period of induction there is a rise in the mitotic rate in this region of the endoderm (Bellairs, 1955). Similarly, Balinsky (1955) reported that the mitotic rate is raised in tissues when they are stimulated to form a limb. He also found that there is a high rate of division in the regions capable of inducing limb development.

The present paper consists of a study of the mitotic rate in the mesoderm of the young chick embryo to see if the tissue which is believed to bring about foregut induction also has a high mitotic rate.

MATERIAL AND METHODS

I have used the ten specimens that I previously studied for the mitotic rate in the endoderm. Four were at the head-process stage, two at the head-fold stage, three were embryos with 4–5 pairs of somites, and the remaining embryo had 12 pairs of somites (see Bellairs, 1955, for detailed descriptions of these specimens).

Estimations of the mitotic rate of the mesoderm have been made on every third section throughout the whole area pellucida; these were the same sections used for the endoderm counts and they were studied at a magnification of $\times 500$ with an oil-immersion lens. Three main longitudinal strips were distinguished in each blastoderm (see Text-fig. 1) and they corresponded with those used for the

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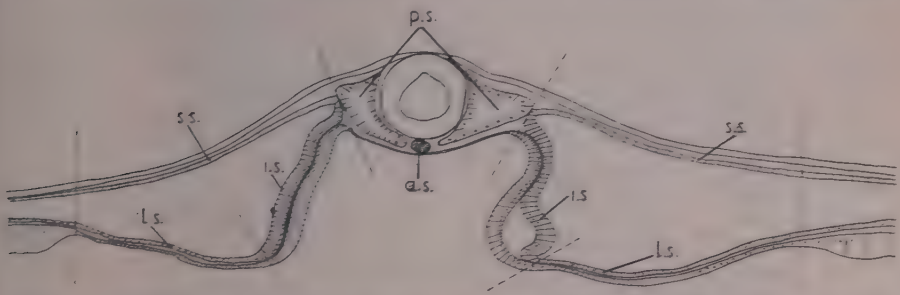
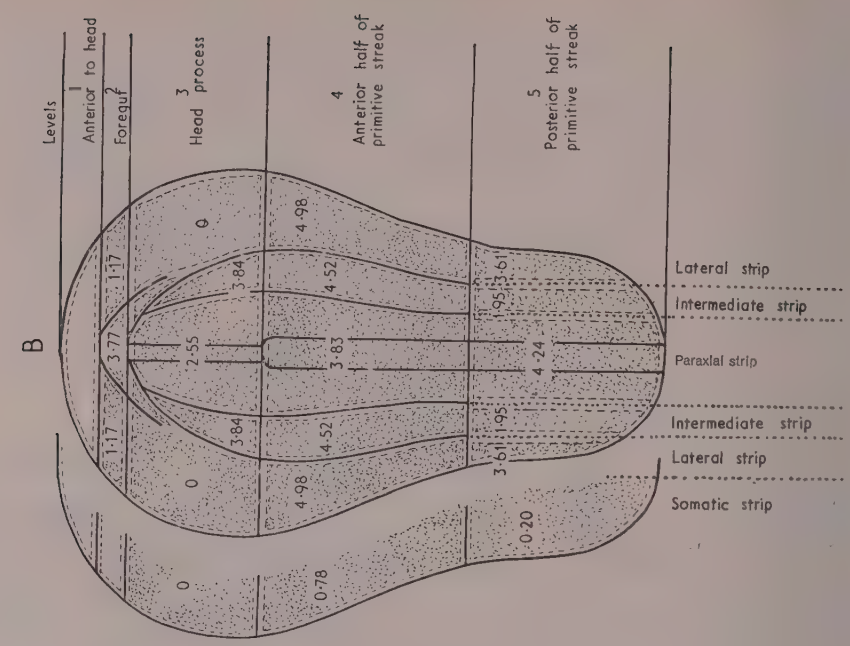
endoderm investigation: (*a*) *Axial mesoderm* lying in the central region and consisting of notochord anteriorly and primitive streak posteriorly. (*b*) *Paraxial strip*, lying above the gut or presumptive gut roof and consisting in the head region of mesenchyme, in the anterior trunk region of somites, and posteriorly of undifferentiated mesoderm. The axial and the paraxial strips together lie above that region of the endoderm which I have previously called the 'central strip' (Bellairs, 1955). (*c*) *Intermediate strip*, the splanchnic mesoderm lying on either side of the central strip in two parts except in the case of a closed foregut

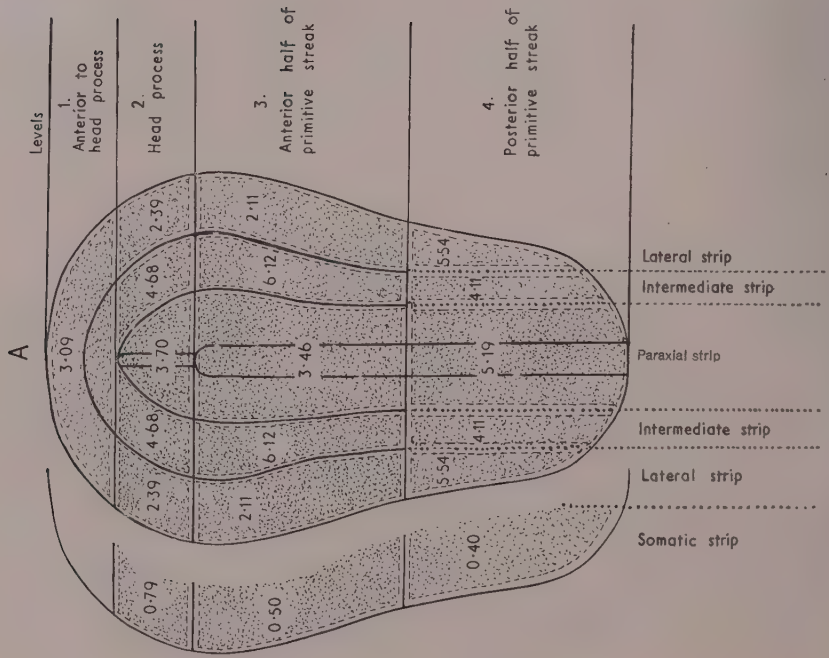


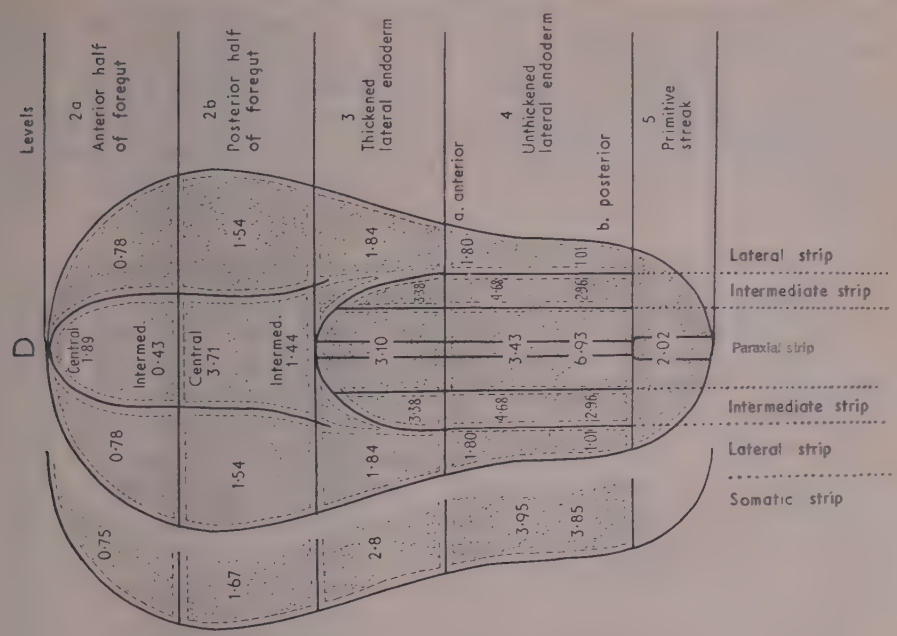
TEXT-FIG. 1. Diagram showing the appearance of the various mesodermal strips in a typical transverse section. *a.s.*, axial strip; *p.s.*, paraxial strip; *i.s.*, intermediate strip; *l.s.*, lateral strip; *s.s.*, somatic strip. The broken lines indicate the boundaries of the strips.

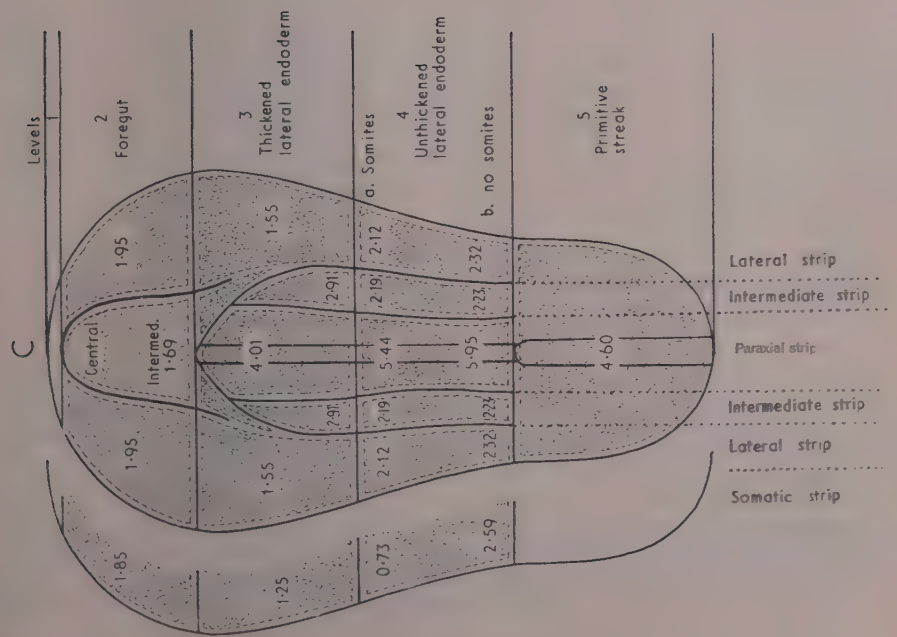
where the two parts are united with each other below the gut; it is thicker at the anterior than the posterior end and where it is thick it lies close against the thick endoderm of the developing foregut (Text-fig. 1). In the somite stage embryos it includes the epimyocardial rudiment of the heart. (*d*) *Lateral strip*, the splanchnic mesoderm lying in two parts lateral to the intermediate strip mesoderm except in the case of a closed foregut where the two parts form a continuous covering over the yolk sac. It is usually considerably thinner than the intermediate strip mesoderm. Towards the hind end in some of the embryos and throughout most of one of the head-fold stage embryos, it was not possible to distinguish critically the junction between regions (*c*) and (*d*). In these cases the mitotic counts for the two regions were made together. (*e*) *Somatic strip* lying dorsal to strips (*c*) and (*d*) and consisting of a thin sheet of somatic mesoderm.

The mitotic rate was taken to be (number of dividing cells ÷ number of resting cells) × 100. The counts for both resting and dividing cells obtained for each strip were grouped into levels (see Text-fig. 2) according to the antero-posterior position of the sections in the blastoderm. The levels were determined on morphological grounds and were the same as those used previously in the endoderm assessments, so that it was possible to compare the mitotic rate of the endoderm and mesoderm in the same region. In the somite stage specimens level 4 was treated as an anterior part (4*a*) and a posterior part (4*b*). In specimens 7, 8, and 9 the border between levels 4*a* and 4*b* was the posterior end of the somite series.


B
Levels
1 Anterior to head
Foregut
2
3 Head process
4 Anterior half of primitive streak
5 Posterior half of primitive streak
Lateral strip
Intermediate strip
Paraxial strip
Intermediate strip
Lateral strip
Somatic strip
1·17
3·77
2·55
3·84
4·98
4·52
3·83
1·95
3·61
4·24
0·20
0·78


A
Levels
1. Anterior to head process
2. Head process
3. Anterior half of primitive streak
4. Posterior half of primitive streak
Lateral strip
Intermediate strip
Paraxial strip
Intermediate strip
Lateral strip
Somatic strip
3·09
2·39
4·68
3·70
2·11
6·12
3·46
5·54
4·11
5·19
0·40
0·50
0·79


D
Levels
2a Anterior half of foregut
2b Posterior half of foregut
3 Thickened lateral endoderm
a. anterior
4 Unthickened lateral endoderm
b. posterior
5 Primitive streak
Central 1·89
Intermed. 0·43
Central 3·71
Intermed. 1·44
0·78
1·54
1·84
1·80
4·68
3·38
3·10
3·43
6·93
2·96
1·01
2·02
0·75
1·67
2·8
3·95
3·85
Lateral strip
Intermediate strip
Paraxial strip
Intermediate strip
Lateral strip
Somatic strip


C
Levels
2 Foregut
3 Thickened lateral endoderm
a. Somites
4 Unthickened lateral endoderm
b. no somites
5 Primitive streak
Central
Intermed. 1·69
1·95
1·55
2·12
2·32
2·19
2·23
2·91
4·01
5·44
5·95
4·60
1·85
1·25
0·73
2·59
Lateral strip
Intermediate strip
Paraxial strip
Intermediate strip
Lateral strip
Somatic strip

5584.4

A a

In specimen 10 somites are present through most of level 4 so that it was simply divided into an anterior half (level 4*a*) and a posterior half (level 4*b*).

The χ^2 test was used for estimating the statistical significance of the differences in mitotic rate between different regions in each specimen; a probability of 5 per cent. was used as the level of significance. Where any of the expected values were less than 5 the χ^2 test was considered to be unreliable and was discarded. The correction for continuity was made.

RESULTS

The mitotic rates for the area pellucida mesoderm were highest in the head-fold stages. The overall mean rates for the individual embryos were: head-process stage, 3·87, 3·24, 3·55, 3·67; head-fold stage, 4·06, 5·68; somite stages 1·96, 3·24, 1·80, 2·64.

As in the previous paper attention has been concentrated on the differences which exist in the mitotic rates between different parts in each embryo. The results of the analysis fall into two groups: (*a*) the differences in the mitotic rate between the various parts of the mesoderm, (*b*) the relationships between the mitotic rates in certain areas of the mesoderm and in the endodermal tissues next to them.

Text-fig. 2 shows the mitotic rates in the mesoderm of four specimens, one taken from each group. Table 1 is of the mitotic rates in the various parts of the mesoderm in each of the four somite-stage specimens.

(*a*) *Comparison of the mitotic rates in different parts of the mesoderm*

The main results obtained were that the highest rates of mitosis were consistently found in either the intermediate mesoderm or the paraxial mesoderm. In every specimen, except that of the 12-somite stage, the intermediate mesoderm at level 3 or level 4 (in both levels in 7 cases) had a mitotic rate significantly higher than that in the somatic mesoderm at the same level (Table 2). In no case did the somatic mesoderm have a mitotic rate significantly higher than that in the intermediate mesoderm at any level.

Similarly, in either level 3 or level 4 (in both levels in 4 cases) of these same specimens the paraxial mesoderm had a significantly higher rate of division than the somatic mesoderm (Table 3). In no case was the reverse situation true.

TEXT-FIG. 2. Diagram representing the mesoderm of the four stages. A, head-process; B, head-fold; C, embryo with 4 pairs of somites; D, embryo with 12 pairs of somites. Each is considered as a number of antero-posterior levels and medio-lateral strips whose limits are based on morphological features. The mesoderm is thus represented as a number of regions, the positions of each being determined by the antero-posterior level and the medio-lateral strip in which it lies. These regions are outlined by broken lines. Where the foregut has begun to form as in B, C, D, the paraxial strip (head mesenchyme) is obscured in the diagram by the intermediate strip (splanchnic mesoderm). The axial strip (notochord and primitive streak) is not represented in the diagram. The mitotic rates are given for 4 representative specimens, A, specimen 3; B, specimen 5; C, specimen 8; D, specimen 10.

The lateral mesoderm though usually thicker than the somatic mesoderm is also a thin sheet of cells. In 6 out of 9 cases where it was possible to make comparisons, the mitotic rate in the intermediate (splanchnic) mesoderm was found to be significantly higher than that in the lateral (splanchnic) mesoderm in at least one level (Table 4). In no case did the lateral mesoderm have a significantly

TABLE 1

The mitotic rates expressed per 100 intermitotic cells obtained in the different regions (see Text-fig. 2) of the four 'somite-stage' embryos. The number of dividing cells is given in brackets in each case. Total is the overall rate for the whole area pellucida mesoderm

Specimen	*Level*	*Paraxial strip*	*Intermediate strip*	*Lateral strip*	*Somatic strip*	*Axial strip*	*Total*
7	2	0·39 (3)	0·24 (1)	0 (0)	0 (0)		
	3	0·99 (18)	1·26 (34)	0·44 (5)	0·36 (5)		
	4*a*	2·30 (23)	2·27 (29)	0·16 (1)	0·34 (2)	1·89 (15)	1·96
	4*b*	2·81 (64)	0·90 (16)	0·19 (3)	Included with *b* and *c*		
	5	2·33 (159)					
8	2	3·77 (9)	1·69 (6)	1·95 (19)	1·85 (5)		
	3	4·01 (58)	2·91 (65)	1·55 (10)	1·25 (15)		
	4*a*	5·44 (50)	2·19 (25)	2·12 (9)	0·73 (5)	2·17 (20)	3·24
	4*b*	5·95 (107)	2·23 (18)	2·32 (13)	2·59 (20)		
	5	4·60 (315)					
9	2	2·38 (43)	2·74 (12)	0·77 (2)	1·17 (3)		
	3	3·47 (44)	1·79 (31)	1·32 (8)	0·47 (5)		
	4*a*	2·37 (61)	2·11 (38)	0·92 (12)	0·47 (7)	1·15 (12)	1·80
	4*b*	2·59 (37)	2·42 (21)	0·33 (2)	0·12 (1)		
	5	1·90 (195)					
10	2*a*	1·89 (140)	0·43 (4)	0·78 (6)	0·75 (8)		
	2*b*	3·71 (72)	1·44 (40)	1·54 (15)	1·67 (19)		
	3	3·10 (34)	3·38 (65)	1·84 (8)	2·8 (22)	1·28 (7)	2·6
	4*a*	3·43 (144)	4·68 (126)	1·80 (22)	3·95 (92)		
	4*b*	5·92 (85)	2·96 (27)	1·01 (7)	3·85 (66)		
	5	2·02 (66)					

higher mitotic rate than the intermediate mesoderm. Similarly, in 6 out of 9 cases the mitotic rate in the paraxial mesoderm was found to be significantly greater than that in the lateral mesoderm at one or more levels (Table 5). In no case did the lateral mesoderm have a significantly higher mitotic rate than the paraxial mesoderm.

The mitotic rate in the axial strip (head process or primitive streak) was in no case found to be significantly greater than that in the paraxial mesoderm adjacent to it; in three specimens it was actually found to be significantly lower.

(b) The mitotic rate of the mesoderm compared with that of the endoderm

In every specimen the overall mean rates for the mesoderm were significantly higher than the corresponding ones for the endoderm (given by Bellairs, 1955). When different regions of the mesoderm were compared individually with the

TABLE 2

χ^2 values obtained by comparing mitotic rates in intermediate mesoderm and somatic mesoderm of the same level

	Specimen									
Level	1	2	3	4	5	6	7	8	9	10
2	—	**23·88**	**10·11**	—	—	—	—	0·19	1·09	*2a*: 0·09 *2b*: 0·09
3	**6·00**	**74·67**	**55·50**	**56·36**	—	**44·03**	**8·02**	**8·92**	**8·80**	0·56
4*a*	**20·76**	**39·15**	**18·59**	**1·19**	**51·47**	**67·00**	**9·43**	**5·62**	**32·00**	1·31
4*b*							—	0·19	**15·58**	1·29
5	..	..	..	..	**21·55**	**72·5**	..	..	..	..

Significant values of χ^2 (over 5) are shown in heavy type. In each of these cases the higher rate of mitosis occurred in the intermediate mesoderm. In some regions there were no dividing cells (e.g. somatic region of specimen 1, level 2) so that no comparison was possible (indicated as —).

TABLE 3

χ^2 values obtained by comparing mitotic rates in paraxial mesoderm and somatic mesoderm of the same level

	Specimen									
Level	1	2	3	4	5	6	7	8	9	10
2	—	**30·14**	**6·84**	—	—	—	—	—	0·95	*2a*: **6·91** *2b*: **11·61**
3	**74·48**	**46·97**	0·08	**84·67**	—	**39·17**	**4·49**	**17·51**	**24·22**	0·13
4*a*	1·39	**37·63**	**25·11**	1·59	**44·30**	**53·91**	**8·08**	**25·05**	**20·22**	1·09
4*b*							0·00	**11·88**	**16·16**	**5·72**
5	..	..	..	..	**50·82**	**40·47**	..	..	..	..

Significant values of χ^2 (over 5) are shown in heavy type. In each of these cases the higher rate of mitosis occurred in the paraxial mesoderm. Where no comparison was possible, this is indicated as —.

regions of endoderm which underlay them, it was found that the greatest differences were in the intermediate strips. Because of difficulties in defining the limits of one or other of these regions it was possible to make the comparisons in only 8 specimens (3 head-process, 1 head-fold, 4 somite stages, see Table 6). The results fall into a series. In six specimens (3 head-process, 1 head-fold, and 2

Table 4

χ^2 values obtained by comparing mitotic rates in intermediate and lateral mesoderm of the same level

	Specimen									
Level	1	2	3	4	5	6	7	8	9	10
2	—	**6·90**	4·62	—	—	—	—	0·09	2·15	2*a*: 0·05 2*b*: 0·04
3	0·04	1·44	**13·03**	**5·36**	—	—	**5·5**	3·42	1·75	2·63
4*a*	1·79	0·42	1·60	1·68	0·48	—	**12·03**	0·02	**14·98**	**17·48**
4*b*							**5·88**	0·01	0·85	**6·88**
5	..	..	..	..	0·31	—	..	..	..	..

Significant values of χ^2 (over 5) are shown in heavy type. In each of these cases the higher rate of mitosis occurred in the intermediate mesoderm. Where no comparison was possible, this is indicated as —.

Table 5

χ^2 values obtained by comparing mitotic rates in paraxial and lateral mesoderm of the same level

	Specimen									
Level	1	2	3	4	5	6	7	8	9	10
2	—	3·11	1·20	—	—	—	—	—	1·99	2*a*: 4·78 2*b*: **9·98**
3	**12·30**	1·26	0·80	**22·5**	—	—	2·63	**8·04**	**10·4**	1·74
4*a*	0·001	0·50	0·47	2·97	2·41	—	**10·64**	**7·48**	**15·00**	**7·94**
4*b*							0·00	**10·92**	**10·11**	**24·89**
5	..	..	..	..	0·19	—	..	..	..	..

Significant values of χ^2 (over 5) are shown in heavy type. In each of these cases the higher rate of mitosis occurred in the paraxial mesoderm. Where no comparison was possible, this is indicated as —.

Table 6

χ^2 values obtained by comparing mitotic rates in the intermediate mesoderm and intermediate endoderm of the same level

	Specimen							
Level	1	2	3	5	7	8	9	10
2	—	**9·29**	**5·79**	0·43	(4·00)	0·01	**7·22**	**15·74**
3	**14·85**	**9·14**	**24·05**	**8·11**	**7·38**	2·50	0·16	**6·10**
4 (*a*+*b*)	1·33	**11·68**	**7·21**	**67·50**	**7·96**	3·44	3·17	**40·75**

Significant values of χ^2 (over 5) are shown in heavy type. In each of these cases the higher rate of mitosis occurred in the intermediate mesoderm, with the exception of specimen 10; in this embryo the intermediate endoderm had a significantly higher mitotic rate in each level. Where no comparison was possible, this is indicated as —.

somite stages) at least one level and frequently all, had a significantly higher rate of mitotis in the intermediate mesoderm than in the underlying endoderm; in one somite-stage specimen the mitotic rate in the intermediate mesoderm did not differ significantly from that in the intermediate endoderm at any level whilst in the oldest specimen the mitotic rate of this mesoderm was significantly lower than that of the corresponding endoderm in every level.

When the mitotic rate in the lateral mesoderm was compared with that in the underlying endoderm, however, the regions where it was significantly higher were found to be almost confined to level 4 (5 cases out of 9) and in only one of these was a significantly higher mitotic rate found anterior to this (region 3 of a head-process specimen). In the remaining specimens there was no significant difference between the mitotic rates in the lateral mesoderm and lateral endoderm at any level.

DISCUSSION

Derrick (1937) and Schultz (1922) both found a higher rate of cell-division in the entire mesoderm of young chick embryos than in the entire endoderm. This was not only confirmed in the present investigation, but the difference was found to be statistically significant in every specimen. The figures I have obtained are of the same order as those given by Derrick for the head-process and head-fold stages, but the mitotic rate (4·72 per cent.) which she gave for her 5-somite-stage specimen was somewhat higher. The variations which occur between different specimens are well known and it has previously been suggested that as development proceeds, in the early stages the rate of cell-division fluctuates (Derrick, 1937; Bellairs, 1955). This could account for the differences in actual mean rates obtained by different observers, although the existence of such fluctuations in the rat embryo has recently been denied by Corliss (1953).

The present investigation provides no evidence for a high rate of cell-division in the head-process or in the primitive streak, and this, too, is in agreement with Derrick. Similarly, Corliss (1953) found that the mitotic rate in the primitive streak of the rat was no higher than in any other region.

One of the two regions which consistently had a higher mitotic rate than the somatic or lateral mesoderm was the paraxial strip. It seems possible that the high rate of cell-division here was related to the formation and further development of the somites.

One of the main results of the investigation is that relatively high rates of cell-division are found in the intermediate strip. This is the region which is thought to induce the underlying endoderm to form the floor of the foregut. Both tissues become thickened and closely apposed during this time. Like the mesoderm the endoderm also undergoes an increase in the mitotic rate in this region. Thus when the foregut is developing there is a high rate of cell-division in both the inducing and reacting tissues, which is comparable with the situation reported

by Balinsky (1955) for limb induction. It may well be that a high rate of cell-division is characteristic of many tissues taking part in embryonic induction, although this is apparently not so in the head-process and primitive streak which bring about neural-plate induction. Balinsky does not, of course, regard this high mitotic rate as being responsible for inductions, but rather as an activity which accompanies them. My results support this idea as far as the splanchnopleure is concerned.

In the present experiments the changes in thickness and the increase in the mitotic rate begin earlier in the intermediate mesoderm (head-process stage) than in the intermediate endoderm (head-fold and somite stages). It is possible that the physiological changes associated with embryonic induction begin earlier in the inducing tissue than in the induced. If so, then any influence which these may have on cell-division would be expected to appear earlier in the mesoderm than in the endoderm. This is reflected in the fact that in most of the younger specimens the mitotic rate was significantly higher in at least one and frequently in several regions of the intermediate strip of mesoderm than in the endoderm beneath it. It is, however, not clear why the lateral mesoderm should in some cases possess a significantly higher mitotic rate than the underlying endoderm in level 4. It is possibly associated with the fact that the vitelline veins will develop in this region at a later stage.

The high mitotic rate in the intermediate strip mesoderm appears to be correlated with the thickening which takes place in this tissue. Like the intermediate strip endoderm (Bellairs, 1955) this mesoderm appears to get thicker mainly by a change in shape of the individual cells, from relatively flattened to cubical and then to columnar. The change in height of the individual cells composing the epithelium is necessarily accompanied by an increase in the area of contact with the neighbouring cells. It is in those regions where the amount of contact between cells appears to be increasing that high rates of mitosis were found. In the splanchnic mesoderm the thin lateral strips had low mitotic rates, whereas the thickening intermediate strips usually had higher ones; in 6 specimens significant differences were found between the two. It is suggested, therefore, that in an epithelium of this type the process of increasing the amount of contact between cells may stimulate in some way an increase in the rate of mitosis, or alternatively a higher mitotic rate may lead to an increased contact between cells. There is already considerable evidence in the literature (summarized by Hughes, 1952) that the cortex of a cell plays an important role in the process of cell division. If the rate of cell-division were uniform throughout the embryo, then the surface area of the thickening sheet would tend to be reduced relative to the rest of the embryo. This could be prevented by an increase in the volume of the individual cells and/or by an increase in the mitotic rate in such regions. It is not known whether any change in volume of individual cells occurs, but it seems probable that the increased rate of mitosis plays a large part.

SUMMARY

1. Mitotic counts have been made in the area pellucida mesoderm of 10 chick embryos. The significance of the results obtained has been tested statistically.

2. The primitive streak and head process were never found to have a mitotic rate significantly greater than the regions on either side of them.

3. A consistently high mitotic rate was found in thickening splanchnic mesoderm, and in the paraxial mesoderm where somites form.

4. The thickening splanchnic mesoderm is believed to induce thickening of the foregut floor. Both tissues have a high rate of mitosis. This is comparable with Balinsky's (1955) report that both the inducing and induced tissues in limb formation have high mitotic rates.

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Further Studies in Tissue Homotransplantation in Cattle

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INTRODUCTION

THE work which forms the subject-matter of this communication is mainly complementary to that reported upon previously (see Anderson, Billingham, Lampkin, & Medawar, 1951; Billingham, Lampkin, Medawar, & Williams, 1952). It may be recalled that these transplantation studies were initiated in the mistaken belief that the one- or two-egg origin of twins in cattle could be established unequivocally according to the fate of skin grafts mutually exchanged between them, as it can in man (Rogers & Allen, 1955; Snyderman, Rogers, & Allen, 1956). In the event it proved otherwise, for although cattle reacted against and destroyed with great promptitude skin homografts from unrelated individuals, or even from full siblings of separate birth or from their dams, skin grafts exchanged between dizygotic twins nearly always proved to be almost as acceptable as those exchanged between monozygotic twins.

It was suggested at the time that the mechanism responsible for this highly specific 'tolerance', or inability of dizygotic twins to react against grafts of each other's skin, was the same as that which underlies their persistent red cell chimaerism (Owen, 1945; Burnet & Fenner, 1949): a mutual exchange of cells, including red cell precursors, during foetal life made possible by the existence of anastomoses between the blood circulations of the embryos (Tandler & Keller, 1911; Lillie, 1916, 1917). This suggestion has now been confirmed by experiments in which various laboratory mammals and birds were inoculated with living homologous tissue-cells in embryonic life. Then, after the animals had grown up and attained immunological maturity, they were found to be fully tolerant of skin homografts transplanted to them from the original donors, or from other donors of similar genetic constitution (see Billingham, Brent, & Medawar, 1953, 1955, 1956*a*). More pertinent is the fact that in chickens where persistent red cell chimaerism also occurs naturally in dizygotic twins and can be

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brought about experimentally by employing Hašek's (1953*a*) technique of parabiosing embryos in such a way that vascular anastomoses are established between them, it is likewise accompanied by tolerance of skin homografts (Billingham, Brent, & Medawar, 1956*a*).

The experiments now to be described were carried out to investigate (i) whether the previously demonstrated differential response of dizygotic twins towards grafts from their dam (Billingham *et al.*, 1952) can be taken as valid evidence of their two-egg origin, (ii) whether skin grafting may be used as a reliable method for early diagnosis of the freemartin condition, (iii) the responses of tetrazygotic quadruplets to grafts of their dam's skin, (iv) possible consequences of an accidental exchange of cells between a bovine foetus and the dam, (v) the influence of pregnancy on the homograft reaction in cattle, and (vi) whether new-born calves can react against homografts.

METHODS

The skin grafts were removed from the dorsa of the donors' ears and transplanted to the withers of the recipients as in our previous studies. The standard dosage of skin transplanted to a recipient from any single donor comprised four fitted 'pinch' grafts. In addition, to provide a control for the technical success of a grafting operation, four skin autografts—i.e. grafts of the recipient's *own* skin—were transplanted concomitantly. Full details of the operative procedures and dressings applied have already been described. The survival times of the homografts were assessed on the basis of careful inspection of the grafts *in situ* at regularly spaced intervals, confirmation being obtained where necessary by the histological examination of biopsy specimens.

RESULTS

(i) *Transplantation of skin from dams to their monozygotic twin calves*

In each of four trials carried out previously it was found that grafts from a dam elicited significantly different reactions from her two-egg twin calves. To find out whether this asymmetry of response can be taken as evidence of the two-egg origin of the twins, the responses of three sets of twins, classified by ordinary methods of phenotypic appraisal as monozygotic, to grafts of their dam's skin have now been studied. These tests are set out in Table 1.

As anticipated on theoretical grounds, there was a remarkable similarity between the individual twins' reactions to their grafts. The difference of 2 days between the survival times of the grafts on the third pair of twins could easily be accounted for by slight physiological differences during the early stages of healing, originating from unavoidable variation in surgical technique (see Billingham, Brent, Medawar, & Sparrow, 1954). The survival times of all the grafts fell within the range obtained for homografts transplanted from a dam to a calf of single birth, or between full siblings of separate birth. Unlike grafts

transplanted from a dam to dizygotic calves, there was no evidence that the survival of any of the present series of grafts had been prolonged.

In addition to the tests described above, we have also investigated the responses of two more sets of dizygotic twins (these were of unlike sex) to grafts from their dams. These tests also are included in Table 1. In one pair there was a conspicuous asymmetry of response comparable with our previous findings and, as Table 2 shows, these animals were intolerant of grafts exchanged between them. The other pair of animals were highly tolerant of each other's grafts.

TABLE 1

Transplantation of grafts from dam to twin calves. The first three pairs of twins were classified as monozygotic, the last two pairs were dizygotic

Dam	Recipients (calves)	Sire	At operation		Homograft survival time	See also
			Age	Weight		
			(*days*)	(*lb.*)	(*days*)	
A.	MZ 154A♀	Fr.	98	166	9	..
	MZ 154B♀			158	9	..
J × Sh.	MZ 157A♀	A.	83	102	9	..
	MZ 157B♀			166	9	..
A.	MZ 159A♀	A.	39	66	10	..
	MZ 159B♀			60	12	..
Fr. · Sh.	BH 17A♀	Fr. ×	911	984	10	Table 2
	BH 17B♂			1,150	11	
J. .	ABGZ 21♀	J.	26	67	> 7 < 9	Table 2
	ABGZ 21♂			61	> 9 < 12	

Abbreviations of breed names: A., Ayrshire; J., Jersey; Fr., Friesian; Sh., Shorthorn.

The present findings with monozygotic twins suggest that when a clearly distinguishable asymmetry of response of cattle twins towards grafts of their dam's skin is demonstrable, it may be accepted as valid evidence of their dizygous origin.

(ii) *The use of skin grafting to distinguish between freemartins and sexually normal females*

Since the tolerance which dizygotic twins normally exhibit towards grafts of each other's skin is a consequence of an anastomosis between their circulations in foetal life, it follows that dizygotic twins which were not so united *in utero* should not be tolerant of each other's grafts. Now as vascular intercommunion in foetal life is also a necessary prerequisite for the reproductive abnormality of females born co-twin with males (i.e. 'freemartins', see Lillie, 1917; Swett, Matthews, & Graves, 1940), exchange of skin with their male partners should discriminate between freemartins and sexually normal females.

TABLE 2

Reciprocal exchange of grafts between females born co-twin with males and their male partners

Donor	Recipient	At operation: Age (days)	At operation: Weight (lb.)	Observation period (days)	Appearance of reaction after operation (days)	Notes
BH 5B♂	BH 5A♀	483	695	179	..	Freemartin.
BH 5A♀	BH 5B♂		648		..	..
BH 7B♂	BH 7A♀	444	631	169	..	Freemartin.
BH 7A♀	BH 7B♂		670		69	Transient inflammatory reaction; grafts recovered.
BH 8B♂	BH 8A♀	444	302	188	..	Freemartin.
BH 8A♀	BH 8B♂		714		..	..
BH 9B♂	BH 9A♀	365	662	109	85	Freemartin. Grafts broke down by 109th day.
BH 9A♀	BH 9B♂		634	191	109	Persistent chronic inflammatory reaction.
BH 13B♂	BH 13A♀	291	630	..	14	Fertile; has calved.
BH 13A♀	BH 13B♂		620		14	Breakdown of grafts on both animals complete by 17th day.
BH 16B♂	BH 16A♀	245	368	194	..	Freemartin.
BH 16A♀	BH 16B♂		372		..	..
BH 17B♂	BH 17A♀	183	236	192	149	Freemartin. Persistent chronic inflammatory reaction.
BH 17A♀	BH 17B♂		250		..	..
BH 20B♂	BH 20A♀	464	592	332	..	Probably a freemartin. Not observed to come on heat.
BH 20A♀	BH 20B♂		674		98	Persistent chronic low-grade reaction.
BH 24B♂	BH 24A♀	376	548	98	..	Freemartin.
BH 24A♀	BH 24B♂		560		..	..
BH 25B♂	BH 25A♀	408	477	229	70	Freemartin; persistent chronic reaction.
BH 25A♀	BH 25B♂		434		$> 97 < 229$	Persistent chronic reaction.
BH 26B♂	BH 26A♀	325	460	230	..	Freemartin.
BH 26A♀	BH 26B♂		498		..	..
BH 27B♂	BH 27A♀	325	492	232	..	Freemartin.
BH 27A♀	BH 27B♂		512		..	..
BH 30B♂	BH 30A♀	247	370	232	..	Freemartin.
BH 30A♀	BH 30B♂		400		..	..
BH 31B♂	BH 31A♀	235	385	..	85	Freemartin; transient reaction which subsided by 106th day.
BH 31A♀	BH 31B♂		388	..	64	Complete destruction of grafts by 106th day.
BH 32B♂	BH 32A♀	204	302	232	..	Freemartin.
BH 32A♀	BH 32B♂		296		..	..
HB 1B♂	HB 1A♀	52	125	146	..	Freemartin.
HB 1A♀	HB 1B♂		113		..	..

Donor	Recipient	At operation: Age	At operation: Weight	Observation period	Appearance of reaction after operation	Notes
		(*days*)	(*lb.*)	(*days*)	(*days*)	
HB 2B♂	HB 2A♀	55	101	146	..	Freemartin.
HB 2A♀	HB 2B♂		140		..	..
HB 3B♂	HB 3A♀	51	119	120	..	Freemartin.
HB 3A♀	HB 3B♂		120		..	..
HB 4B♂	HB 4A♀	51	97	120	..	Freemartin.
HB 4A♀	HB 4B♂		126		..	..
HB 5B♂	HB 5A♀	51	130	120	..	Freemartin.
HB 5A♀	HB 5B♂		136		..	..
HB 6B♂	HB 6A♀	164	257	337	..	Freemartin.
HB 6A♀	HB 6B♂		301		..	..
B 61♂	MZ 61A♀	52	109	103	..	Freemartin.
MZ 61A♀	B 61♂		116		..	..
B 61♂	MZ 61B♀		114		..	Freemartin.
MZ 61B♀	B 61♂		116		..	..
TZ 1D♂	TZ 1A♀	190	208	240	..	Freemartin.
TZ 1A♀	TZ 1D♂		225		..	..
TZ 1D♂	TZ 1B♀		182		..	Freemartin.
TZ 1B♀	TZ 1D♂		225		..	..
TZ 1D♂	TZ 1C♀		224		106	Freemartin; persistent chronic reaction culminating in graft destruction by 240th day.
TZ 1C♀	TZ 1D♂		225		..	..
ABGZ 20♂	ABGZ 21♀	25	67	..	9	Fertile; has calved. Breakdown of grafts by 12th day.
ABGZ 21♀	ABGZ 20♂		61	..	9	Breakdown of grafts by 16th day.

NOTES: MZ 61A, MZ 61B, and B 61 were triplets, of which MZ 61A and B were females and almost certainly monozygous. B 61 was a male.

TZ 1A, TZ 1B, TZ 1C, and TZ 1D were quadruplets and almost certainly tetrazygotic. TZ 1D was a male, the remainder being females.

To test this possibility grafts have been exchanged between the members of twenty-two sets of bull/heifer twins of various breeds. In addition, graft interchanges were made between the male and two female members of a set of triplets (B 61 and MZ 61 A and B) in which the females had been classified as monozygotic, and between the male and three female members of a set of quadruplets (TZ 1 A, B, C, and D) believed to be tetrazygotic. It will be seen from Table 2 that the ages of these animals at the time of operation ranged from 25 to 483 days. All the test grafts were kept under observation for a considerable period. The condition of the reproductive organs of the twenty-seven females grafted—i.e. whether they were freemartins—was established on the basis of a careful clinical examination by a veterinary surgeon when they were old enough or, in a few cases, directly by *post mortem* examination.

As Table 2 shows, 25 of these 27 females were highly tolerant of their male partners' grafts, for there was no outward indication that any of them had begun to react against its partner's grafts before the 70th post-operative day, and in the 5 in which there was subsequent evidence of a reaction, it was so weak that it culminated in the destruction of the grafts on only a single animal. All these highly tolerant animals were found to be freemartins. The remaining two females, in which no abnormalities of their reproductive organs could be detected, reacted with normal vigour against their grafts and have now established their fertility beyond question by giving birth to calves. The very small proportion of normal females in this series, only 2/27 (7·4 per cent.) is consistent with the findings of others on the incidence of normal fertility in females born co-twin with males (see Swett *et al.*, 1940; Stone, Stormont, & Irwin, 1952).

TABLE 3

Analysis of data of Table 2

Age at test operation	*No. of animals*	*No. which reacted against their grafts within 100 days of test operation*	*Percentage of reactors within 100 days*
< 365 days	35	6	17
> 365 days	14	4	28·6

$\chi^2 = 0{\cdot}8041$; $P = 0{\cdot}3$.

With the triplets, only the reactions of the two females towards grafts from their male partner have been considered; with the quadruplets, only the reactions of the three females towards grafts from their male partner have been considered. The males have been deliberately omitted from this analysis since each received grafts from *all* its sisters. It is assumed that BH 25B did not begin to react against its grafts until after they had been in residence for 100 days.

These findings give very strong support to the idea that skin grafting will identify satisfactorily freemartins and sexually normal females.

By grafting from female to male in every pair or group, as well as *vice versa*, we have been able to add to our previous data on the fate of grafts exchanged between dizygotic twins. The present data are particularly important since all the animals between which exchanges were made were of unquestionable dizygosity because they were of unlike sexes, whereas in our previous experiments nearly all the dizygous twins tested were in unisexual sets. Furthermore, in the present experiment it will be seen that seven sets of twins were more than a year old when they were test grafted; the results of these tests carried out on older animals, combined with the very long periods during which the grafts were under observation, provide strong evidence as to the persistence of the tolerant state. Analysis of the data presented in Table 2 (see Table 3) suggests that the probability of a test graft surviving for 100 days after transplantation without there being a hint of a reaction is not significantly less in older animals than in younger ones.

(iii) *Transplantation of skin from a dam to her tetrazygotic quadruplets*

Our previous studies had shown that grafts transplanted from their dams to their dizygotic twin offspring frequently survive for slightly longer than maternal grafts transplanted to calves of single birth. The explanation put forward to account for this prolongation was that, with two-egg twins, each individual is unresponsive to its twin's antigens (as well as to its own) as a result of the prenatal exchange of cells, so that it should be incapable of response to a much wider range of antigens than a calf of single birth. Upon this basis it was predicted that dissimilar triplets should be even less responsive to their dam's grafts and so on. We have now been able to test this prediction by grafting skin from a dam to her 190-day-old quadruplet offspring classified as being of tetrazygotic origin. At the same time grafts were exchanged between the calves, the results of the intersib exchanges being presented in Table 2 (see Section ii). The maternal grafts on these animals were kept under close observation for 70 days. The results are set out in Table 4.

TABLE 4

Transplantation of skin from dam to her quadruplet offspring classified as tetrazygotic

Donor	Recipient	At operation		Condition of grafts at	
		Age	Weight	(*a*) 20 days	(*b*) 70 days
			(*lb.*)		
Dam	TZ 1A♀	190	208	No hint of reaction	Chronic reaction in progress but still fairly high degree of survival.
	TZ 1B♀		182	,,	Chronic reaction far advanced. Low degree of survival.
	TZ 1C♀		224	Very low-grade reaction with perivascular round-cell infiltration of dermis	Chronic reaction far advanced. Low degree of survival.
	TZ 1D♂		225	,,	Low-grade reaction with high degree of survival.

NOTES: All five animals were Ayrshires. For details concerning reactions of calves to grafts exchanged between them, and reactions of dam to grafts from these calves see Tables 2 and 6. All grafts from the dam broke down eventually but the actual survival times were not determined.

Twenty days after the transplantations were carried out the maternal grafts looked perfectly healthy on all four offspring. However, histological examination of biopsy specimens removed at this time gave evidence of an exceedingly weak response on the part of two animals, TZ 1C and D, in the form of diffuse perivascular infiltrations of their grafts with round cells. By the 70th day the outward appearance of their maternal grafts on all four calves indicated that

reactions were in progress. Histological examination confirmed that whereas in two animals breakdown was almost complete, there was still a fairly high degree of survival in the other two. This evidence of asymmetry of response is, of course, not unexpected since the animals were believed to be tetrazygotic. It should be added that the grafts of dam's skin all broke down eventually but the precise survival times were not determined.

As we had predicted and as might have been anticipated from previous findings concerning red cell chimaerism in bovine quintuplets (Owen, Davis, & Morgan, 1946), each of the quadruplets proved to be highly tolerant of the grafts it received from its three partners (see Table 2). During the 240 days these grafts were under observation only one animal, TZ 1C, reacted against the grafts from only *one* of its partners—TZ 1D. The reaction was first noted on the 106th day and was of the chronic type. Graft destruction was not completed until about the 240th day. The behaviour of these quadruplets to grafts of each other's skin is summarized in Table 2.

(iv) *Investigation of the hypothesis that cellular interchange may occur between a dam and her foetus*

An important difference between cattle and all common laboratory mammals is the very long gestation period of the former. One possible immunological consequence of this is the correspondingly greater chance, in cattle, of mother and foetus becoming inoculated with each other's cells through accidental transplacental leakage or diapedesis. If such a process occurred its expected results would be (*a*) in the foetus, provided that maternal cells gained access to it *early* enough, the induction of tolerance which should be demonstrable in later life by a weakened capacity to react against its mother's skin grafts, and (*b*) in the dam, an active immunization, demonstrable by the curtailment of the survival of skin grafts transplanted to her from the offspring after its birth. Some evidence that such a maternally induced tolerance does occasionally occur in guinea-pigs, but not in mice or rabbits which have a much shorter gestation period, has been presented by Billingham, Brent, & Medawar (1956*a*).

With cattle, if such a tolerance induced by leakage of maternal cells does occur but is incomplete, the transplantation of skin from dams to their offspring of multiple birth might possibly give equivocal results. This follows from the finding that with dizygotic twins there is in any case some prolongation of survival of maternal grafts, probably for reasons which have been suggested above (see Section iii). To exclude this possibility we have studied the fate of grafts transplanted from eight dams to their offspring of single birth.

As the results set out in Table 5 show, none of these animals was in any degree tolerant of its test grafts. The present findings may be pooled with those obtained from three previous graftings from dam to calves of single birth (Billingham *et al.*, 1952), and with those from the three trials in which a dam's skin was grafted to each of her monozygous twins (see Section i). Thus, of a total of

seventeen calves of single birth, or members of monozygotic twin pairs, none gave the slightest indication of being tolerant of grafts of its dam's skin.

TABLE 5

Transplantation of grafts from dams to their calves of single birth

Dam	*Breed of dam*	*Calf*	*Sire*	*At operation*		*Homograft survival time*
				Age	*Weight*	
				(*days*)	(*lb.*).	(*days*)
MZ 71A	Fr.	FS 49A	Fr.	283	410	> 8 < 10
MZ 71B	Fr.	FS 49B	Fr.	271	394	> 8 < 10
MZ 81A	Fr.	FS 55A	Fr.	193	325	> 8 < 10
MZ 81B	Fr.	FS 55B	Fr.	176	298	> 8 < 10
MZ 53A	Fr./A.	FS 40A	Fr.	571	750	> 8 < 10
MZ 53B	Fr./A.	FS 40B	Fr.	588	800	> 10 < 12
MZ 54A	Fr./H.	FS 54A	A.A.	219	278	8
MZ 54B	Fr./H.	FS 54B	A.A.	217	291	> 8 < 10

TABLE 6

Transplantation of grafts from calves to their dams

Calf	*Breed of sire*	*Dam*	*Breed of dam*	*Parity of dam*	*At operation*		*Homograft survival time*	*Notes*
					Age	*Weight*		
					(*days*)	(*lb.*)	(*days*)	
FS 49A	Fr.	MZ 71A	Fr.	uniparous	1,070	1,120	> 8 < 10	Animal 210 days pregnant at operation. See also Table 7.
FS 49B	Fr.	MZ 71B	Fr.	..	1,070	1,084	> 8 < 10	..
FS 55A	Fr.	MZ 81A	Fr.	..	1,022	1,104	8	..
FS 55B	Fr.	MZ 81B	Fr.	..	1,022	1,080	6	Immune reaction.
FS 40A	Fr.	MZ 53A	Fr./A.	diparous	1,359	1,048	6	Immune reaction.
FS 40B	Fr.	MZ 53B	Fr./A.	..	1,359	1,026	6	Immune reaction.
FS 54A	A.A.	MZ 54A	Fr./H.	..	1,355	1,152	7	..
FS 54B	A.A.	MZ 54B	Fr./H.	..	1,355	1,160	> 8 < 10	..
TZ 1A	A.	Dam	A.	multiparous	1,400	900	6	Immune reaction. See also Tables 2 and 4.
TZ 1B							8	
TZ 1C							7	
TZ 1D							8	

NOTES: The first two diparous dams received grafts from their first calves; the second two diparous dams received grafts from their second calves; the dam of the quadruplets received grafts from each of her offspring.

Abbreviations of breed names: A., Ayrshire; A.A., Aberdeen Angus; Fr., Friesian; H., Hereford

It was previously found that in two tests in which dams were grafted with skin from their offspring of single birth, destruction of the grafts was so rapid as to suggest that the mother had previously been immunized against (or become

sensitized to) her offspring's cells (see Billingham *et al.*, 1952). Unfortunately, since both dams were multiparous, it was impossible to decide whether the immunization had occurred during the pregnancies of the calves studied. We have now investigated 'natural' immunization of dams more fully by testing some uniparous as well as more multiparous animals (see Table 6).

Of the four uniparous dams tested with grafts from their offspring of single birth, only one behaved as if it had previously been sensitized to its calf's cells. With the 4 diparous dams, 2 were grafted with skin from their first calves and 2 from their second calves. Only 2 of these 4 animals gave evidence of having been immunized. The fact that these were the two which had been test-grafted with skin from their first calves does not necessarily imply that the immunization took place during their first pregnancies, for it is possible that both first and second calves possessed important antigens in common that were lacking in their mother. In this case if cells from either of them gained access to the dam during pregnancy, the immunity elicited might subsequently curtail the life of grafts from either of them.

When the dam of the tetrazygotic quadruplets (see Section iii) was tested with skin from her offspring, evidence of a specific sensitization directed against only one of them was obtained. Unfortunately the fact that the dam had had previous pregnancies leaves open the question of the origin of this sensitization.

Pooling our present findings with those previously obtained, we find that evidence of sensitization of a dam against its offspring's cells has been obtained in 7 out of the 11 animals tested. Moreover, a single pregnancy is sufficient to elicit the phenomenon.

(v) *The influence of pregnancy on the homograft reaction in cattle*

The administration of cortisone or cortisol (hydrocortisone) has been shown to impair the responses of rabbits, guinea-pigs, and mice to skin homografts, resulting in a significant prolongation of their lifetime (Billingham, Krohn, & Medawar, 1951 *a*, *b*; Sparrow, 1953, 1954; Medawar & Sparrow, 1956). Since the output of adrenal corticosteroids is known to increase during pregnancy, Heslop, Krohn, & Sparrow (1954), in experiments conducted on rabbits, sought and obtained evidence that skin homograft survival is prolonged during pregnancy. Grafts transplanted to does between the 20th and 24th days of pregnancy lived about twice as long as normal, though no such prolongation was obtained with grafts transplanted earlier or later. On the basis of these findings these authors suggested that a mother's ability to tolerate her foetus *qua* homograft, without reacting immunologically against it, may depend on the increased cortico-steroid production. The immunological problem of pregnancy has been discussed in detail by Medawar (1953).

Because of their long gestation period—about 283 days—it seemed that the risk of isoimmunization against their foetuses should be much greater in cattle than in rabbits. We have therefore carried out a limited series of trials to see

whether the life of skin homografts is prolonged during pregnancy in this species. Grafts were exchanged between three pregnant cows in such a way that each received skin from the other two. In addition, one animal referred to in Section

TABLE 7

Transplantation of homografts to pregnant cattle

Donors	*Breed of donors*	*Recipient*	*Breed of recipient*	*Duration of pregnancy at operation*	*No. of previous calvings*	*Survival times of homografts*
				(*days*)		(*days*)
ABRO Snowflower	J.	ABRO Annette	Fr.	249	2	$> 8 < 10$
ABRO Janet . .	A.					$> 8 < 10$
ABRO Janet . .	A.	ABRO Snowflower	J.	231	2	$> 8 < 10$
ABRO Annette . .	Fr.					$> 8 < 10$
ABRO Snowflower .	J.	ABRO Janet	A.	203	2	$> 8 \leqslant 10$
ABRO Annette . .	Fr.					$> 8 \ll 10$
FS 49A . . .	Fr.	MZ 71A	Fr.	210	none	$> 8 < 10$

NOTE: In the last entry the pregnant dam was grafted with her calf's skin: see also Table 6.

Abbreviations of breed names: A., Ayrshire; Fr., Friesian; J., Jersey.

iv has been included because it had been grafted with skin from its first calf at a time when it was 210 days pregnant. As Table 7 shows, the survival times of these grafts were all comparable with our controls. Thus, unless it is argued that these tests were carried out either too early or too late, pregnancy does not appear to influence the course of the homograft reaction in cattle.

(vi) *Transplantation of skin homografts to new-born calves*

The faculty of immunological response is known to be feebly developed in young mammals (see Burnet & Fenner, 1949). That new-born calves are to some extent unable to conduct their own immunological defence is implicit in the findings of Kerr & Robertson (1954) with *Trichomonas foetus* antigen, and also by the indispensability of colostrum—containing ready-made antibodies of maternal origin—for their survival (see review by Smith, 1948).

Skin-grafting tests have therefore been carried out to determine whether new-born calves can react against skin homografts (*a*) when they have received, and (*b*) when they have not received colostrum. Eight calves of single birth were given skin homografts from unrelated donors within 15 hours of birth. Four of them received colostrum from their dams; in the remainder it was deliberately withheld, an attempt being made to rear them on milk treated with streptomycin. Unfortunately technical failures of the grafting operations—in our experience always difficult to eliminate with very young calves—in one calf of the first quartet and in two in the second, combined with the death from *B. coli* infection 36 hours after birth of one of the calves which did not receive colostrum, leaves only four survivors.

Despite the small size of the group, the results summarized in Table 8 show clearly that new-born calves can react against skin homografts just as vigorously as adults. The slightly longer survival time of the grafts on SG 5, which was born 3 weeks prematurely, might just conceivably reflect a slight incompleteness of the development of its immunological response mechanism. SG 6's prompt dismissal of its grafts in the absence of colostrum may be taken as evidence that immune serum globulins play no essential role in the homograft reaction, for new-born calves are agammaglobulinaemics (Smith, 1948).

TABLE 8

Transplantation of skin homografts to new-born calves

Calf	*Breed of dam*	*Breed of sire*	*Duration of pregnancy*	*At operation*		*Survival of skin homografts*	*Animal fed on*
				Age	*Weight*		
				(*hr.*)	(*lb.*)	(*days*)	
SG 1	Fr.×J.	A.	286	15	72	$> 8 < 10$	Mother's milk.
SG 2	Fr.×A.	J.	283	11	78	$> 8 < 10$	,,
SG 5	J.	A.	264	$4\frac{1}{2}$	42	$\geqslant 10 < 13$	,,
SG 6	J.	J.	284	13	82	9	Milk from other cows.

NOTE: The normal duration of pregnancy in cattle is 283 days.

Abbreviations of breed names: A., Ayrshire; Fr., Friesian; J., Jersey.

DISCUSSION

The experimental verification that one-egg twins react symmetrically against grafts of their dams' skin may be held to have established the validity of accepting as evidence of dizygotic origin the asymmetries of response which grafts from their dams elicited in four sets of presumed two-egg twins (see Billingham *et al.*, 1952). However, as a diagnostic test, i.e. an exclusion test, for the zygotic origin of cattle twins, skin grafting may occasionally give equivocal results, as reported for example in Section i of the present paper where a bisexual pair of twins reacted symmetrically to their test grafts. For this reason and also because of the skill required in determining the end-point of the homograft reaction with that degree of precision required to establish asymmetries of survival time of only a few days' magnitude, it may prove that blood typing affords a more reliable aid to diagnosis. The drawback of blood typing in cattle, as Hancock (1954) has stressed, is that it is a major undertaking which requires much labour and technical skill.

An extensive series of graft interchanges between females of multiple births and their male partners has shown that only those females which were completely intolerant of their brothers' grafts—i.e. reacted with normal vigour against them—were reproductively normal and became pregnant. All females which exhibited a high, though variable, degree of tolerance of their brothers'

skin grafts manifested abnormalities of their reproductive organs, i.e. were freemartins. Here skin grafting appears to give straightforward and unequivocal results. As a method of differentiating freemartins from reproductively normal females—provided, of course, that the male partner is available—grafting is not inferior to, and just as practicable as the serological method, which depends upon demonstrating the presence or absence of red cell chimaerism (Stone, Stormont, & Irwin, 1952).

Although the evidence presented shows that tolerance of homografts and female sterility are the sequelae of a presumedly common causal condition—vascular interconnexion in foetal life—there are, of course, no grounds for belief that the two phenomena are related as cause and effect. Indeed, the experiments of Billingham, Brent, & Medawar (1956*a*) show very clearly that they are not so: it was found that the fertility was perfectly normal in a series of heterosexual pairs of chickens which, as a consequence of their experimental parabiotic union in embryonic life, had become persistent red cell chimaeras and were also tolerant of each other's grafts (see also Hašek, 1953*b*). Similarly, the fertility was unimpaired in female mice which had previously been inoculated in embryonic life, or within a few hours of birth, with homologous tissue-cells from adult male donors, despite the fact that they had been rendered fully tolerant of grafts from the donor strain and were chimaeras (Billingham, Brent, & Medawar, 1956*a*; Billingham & Brent, 1957).

We have been unable to find any evidence that those freemartins which gave evidence of being incompletely tolerant of their brothers' grafts exhibited less drastic abnormalities of their reproductive systems than those whose grafts gave no evidence of a reaction during the long period during which they were under observation. There is therefore no close correlation between the degree of tolerance of a freemartin towards its brother's grafts and the extent to which its reproductive organs deviate from normal.

As with our previous findings for grafts exchanged between pairs of presumed two-egg twins of similar sex, in the present experiments on bisexual sets of twins whose dizygosity was beyond question, although the majority were highly tolerant of their partners' grafts, evidence of asymmetry of response was obtained. In some pairs only one member reacted against its partner's grafts or, if both reacted, the times of onset or severities of the reactions differed. Unfortunately, the present experiments shed no more light on the aetiology of this asymmetry than did their predecessors. Two factors which either singly or combined may be at least partially responsible are: (1) the genetic or antigenic difference between the individuals. This possibility is sustained by recent findings of Billingham & Brent (1957) that the incidence and quality of tolerance of skin homografts obtained by the intravenous injection of homologous cells into new-born mice depends upon the particular strain combination used. For example, CBA→A is a more favourable combination than A→CBA. And (2) differences between two-egg twins with respect to the completeness with which

the lymphoid—i.e. immunologically responsive (Mitchison, 1953, 1954; Billingham, Brent, & Medawar, 1954)—tissues of each have become invaded or permeated with cells from the other. Although it is known that haematopoietic cells are exchanged prenatally and presumably leave the circulations and settle out in anatomically appropriate places, it is quite possible that there may be considerable individual differences in the extent to which the various lymphoid tissues become chimaeric in a similar manner. As it now seems highly probable that for complete tolerance *all* lymphoid tissues must be adequately exposed to the foreign cells in embryonic life, any nodes which escaped the process of embryonic 'indoctrination' in foetal life might be expected, eventually, to enable their possessor to give a feeble response against the alien cells within its body and, of course, the skin homograft. The long latent period before a reaction appears in some of these animals is perfectly consistent with this concept. An experimental observation which may be relevant here is that when new-born mice of one strain are injected intravenously with a constant dosage of cells from another strain, the degree of tolerance induced in different individuals—even litter mates—in respect of subsequent skin homografts, varies over wide limits, despite the constancy of the genetic disparity between the strains and the most rigid standardization of experimental procedures. It must be added that dizygotic cattle twins are nearly always asymmetrical with respect to the composition of their chimaeric red blood-cell populations (Irwin, 1955).

The sensitization which dams not infrequently exhibit towards their offspring's skin grafts is highly suggestive evidence that cells from the latter may gain access to the mother during their life *in utero* in sufficient numbers to elicit transplantation immunity. Even if it be assumed (*a*) that the sensitization of the dams occurs during pregnancy, and not during the tissue upheavals that underlie parturition, and (*b*) that embryonic tissues are susceptible to transplantation immunity, it is not surprising that this type of maternal sensitization has no apparent ill-effect on the foetus—or its placenta where foetal and maternal tissues are contiguous. Transplantation immunity is mediated by activated cells (Mitchison, 1953, 1954, 1955; Billingham, Brent, & Medawar, 1954; Weaver, Algire, & Prehn, 1955) and a large scale crossing of the placental barrier by the appropriate type of immunologically activated cell would be required to harm the foetus.

The findings that maternal isoimmunization in respect of 'transplantation immunity' antigens (see Billingham, Brent, & Medawar, 1956*b*) does occur in cattle without any apparent adverse effects, and that pregnancy does not prolong the life of homografts in this species may be held to restrict the general validity of Heslop *et al.*'s (1954) suggestion that the increased secretion of adrenal corticosteroids during pregnancy represents a biological mechanism for damping down the mother's capacity to react against her foetus *qua* homograft.

The vigorous response of new-born calves to skin homografts was to be expected from Schinckel & Ferguson's (1953) demonstration that foetal sheep

can react against and destroy within 2 weeks homografts transplanted to them as early as 40 days before birth. These findings are important because they show that immune serum globulins play no essential role in the homograft reaction—ruminants receive no antibodies from their mother during gestation and at birth their plasma contains no serum globulins (Pederson, 1945); they get them in the colostrum from the mother after birth.

SUMMARY

The present contribution presents a sequel to our previous investigations into problems which arose when attempts were made to use skin grafting to differentiate between monozygotic and dizygotic twins in cattle.

1. In each of three trials grafts from a dam elicited similar reactions when transplanted to each of her one-egg twin offspring. On the basis of this finding the asymmetry of response which the majority of two-egg twins show towards grafts from their dam may be taken as evidence of their dizygotic origin.

2. It has been shown that mutual graft exchange between unlike-sexed twins can be used as a reliable method for distinguishing reproductively normal females from freemartins. The complete success of this method provides strong evidence that homograft tolerance and the freemartin condition have a common anatomical origin.

3. Grafts from a dam to her tetrazygotic offspring lived for upwards of 70 days, whereas grafts from a dam to her offspring of single birth rarely live for as long as 14 days. It is argued that this great prolongation in the life of their grafts is the outcome of the quadruplets having previously exchanged cells in foetal life through anastomoses of their circulations. Each must be incapable of reacting against a very wide spectrum of antigens since it will accept grafts from all its brothers and sisters.

4. Evidence is presented which suggests that cells from her foetus not infrequently gain access to a dam and sensitize her to subsequent grafts of her offspring's skin. A first pregnancy may be sufficient to elicit this sensitization. On the other hand, if maternal cells gain access to the foetus, this must occur very rarely for in none of seventeen calves tested with grafts of their dam's skin was there the slightest evidence that they were tolerant.

5. New-born calves react as vigorously as adult cattle against homografts, even when they have not received colostrum. Since new-born calves possess no serum globulins, including antibodies, this provides strong evidence that immune globulins play no essential part in the homograft reaction.

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Embryonic Responses to Structurally Related Inhibitors

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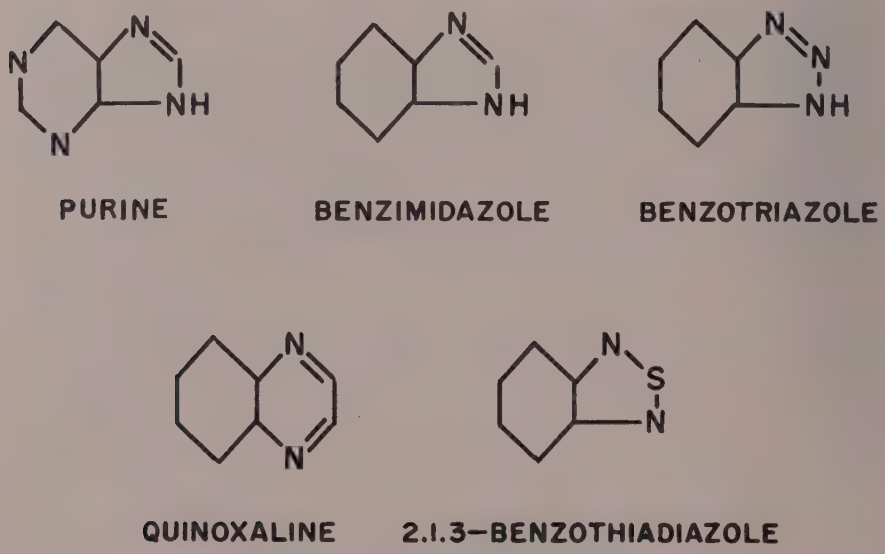
WITH ONE PLATE

INTRODUCTION

MANY of the so-called nucleic acid antagonists inhibit amphibian development (Bieber, 1954; Bieber, Nigrelli, & Hitchings, 1952; Waddington, Feldman, &



TEXT-FIG. 1. Parent structures of inhibitors.

Perry, 1955); a number of them affect embryological processes differentially (Liedke, Engelman, & Graff, 1954, 1955), some of them interfering primarily

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with early cleavage stages where synthesis is not appreciable, others affecting most drastically only late development in which differentiation and synthesis are marked. These compounds, which bear some structural resemblance to the naturally occurring purines, affect other biological systems also, among them *Tetrahymena gelii*, *Escherichia coli*, and tissue cultures of mammalian cells (Gillespie, Engelman, & Graff, 1954).

This paper reports the study of the effects of 35 compounds, substituted benzothiadiazoles, benzimidazoles, quinoxalines, and other related compounds. While 8 of these compounds had been used previously (Liedke, Engelman, & Graff, 1954), 27 are additional new compounds, some of them only newly synthesized. The structures of the parent compounds are illustrated in Text-fig. 1. Since it had been observed in the previously reported compounds as well as in those 27 newly reported (Table 1) that a nitro substituent appeared to enhance inhibitory activity, a number of compounds were selected from both groups for more intensive study and comparison. It appeared provisionally that the nature and position of the substituents rather than the structure of the nucleus, whether benzimidazole, quinoxaline, benzotriazole, or benzothiadiazole, would determine, not only the intensity of inhibition, but also the nature of the inhibition and the morphological response of the embryo.

EXPERIMENTAL

The methods are the same as those described previously (Liedke, Engelman, & Graff, 1954, 1955). Table 1 shows that 17 of the 27 newly tested compounds were inhibitory toward *Rana pipiens* embryos on continuous exposure starting at Shumway (1940) stages 3, 8, 14, or 18 at concentrations of 0·1 mg./ml. excepting where solubility was a limiting factor. Table 2 affords a comparison of the selected compounds at a much lower concentration, 0·01 mg./ml.; the conditions were otherwise the same. The compounds were selected so that they had similar group substitutions on the respective benzothiadiazole, benzimidazole, quinoxaline, or benzotriazole nucleus. Eight of these selected 16 compounds are also listed in Table 1, i.e. they were new ones, while the other 8 had been used in previous experiments (Liedke, Engelman, & Graff, 1954, 1955). Table 2 shows that the substituted compounds are more effective inhibitors than the unsubstituted ones; benzothiadiazole and benzimidazole at low concentrations permit of normal development, but the 5-nitro-substituted compound in each case is strongly inhibitory. Both Tables 1 and 2 show that the benzotriazoles affect late tail-bud stage 18 embryos more severely than they do younger embryos. Two of the benzothiadiazoles, on the other hand, 4-M-6-NBD and 5-NBD, arrest both early and tail-bud stage embryos impartially.

By varying the length of exposure and using a concentration of 0·1 mg./ml. it was found that the time required to stop exposed 2-cell stages in early cleavage was one hour or less for all four benzothiadiazoles and for all four quinoxalines, but it took 3 hours for 4-M-6-NBZ, and indeed 6 hours for 5-NBT, and even 20

hours for 5-NBZ. The other benzimidazoles (BZ, 6-E-4-NBZ) and the benzotriazoles (4-M-6-NBT and 6-E-4-NBT) arrested the embryos only in later stages on exposures up to 20 hours, starting at the 2-cell stage. To stop blastulae (stage

TABLE 1

Stage of arrest in development of Rana pipiens *embryos after continuous exposure to various chemicals, named according to American Chemical Abstracts nomenclature*

(N = Normal development)

Compound	*Reference to synthesis*	*Concentration (mg./ml.)*	*Embryonic stage (S.) at which development stopped when exposed at*			
			Cleavage S. 3	*Blastula S.* 8	*Neurula S.* 14	*Tailbud S.* 18
2,1,3-Benzothiadiazole	(1)	0·1	4–5	12	23	23
6-Methoxy-4-nitro-2,1,3-benzothiadiazole	(1)	0·1	3–5	11–12	19	20
4-Methoxy-6-nitro-2,1,3-benzothiadiazole	(1)	0·1	3–5	11–12	19	20
5-Nitro-2,1,3-benzothiadiazole	(1)	0·1	4	8	14	18
6-Ethoxy-4-nitrobenzimidazole	(2)	0.05	11–12	N	N	..
5-Methoxy-2-methyl-6-nitrobenzimidazole	(2)	0·1	22	22	..	..
6-Amino-2-hydroxy-4-methoxybenzimidazole	(3)	0·1	N	N	N	N
6-Amino-4-methoxy-2-methylbenzimidazole	(3)	0·1	N	N	N	N
6-Amino-5-methoxy-2-methylbenzimidazole	(1)	0·1	N	N	N	N
4-Amino-6-ethoxy benzimidazole	(2)	0·1	N	N	N	N
7-Ethoxy-5-nitroquinoxaline	(2)	0·1	3	12	18	21–23
2,3-Dimethyl-7-methoxy-5-nitroquinoxaline	(3)	0·05	18	18–20	18–20	21–23
2,3-Dimethyl-7-ethoxy-5-nitroquinoxaline	(2)	0·1	18	20	18–20	21–23
2,3-Dimethyl-6-methoxyquinoxaline	(4)	0·1	22	22	..	..
2,3-Diphenyl-6-methoxyquinoxaline	(5)	0·1	N	N	N	N
2,3-Diphenyl-7-methoxy-5-nitroquinoxaline	(6)	0·1	N	N	N	N
6-Ethoxy-4-nitrobenzotriazole	(2)	0·025	4–7	11	14–15	18
6-Methoxy-4-nitrobenzotriazole	(7)	0·025	8–10	11	14–15	19–20
1-Acetyl-5-methoxybenzotriazole	(8)	0·1	8–9	16–17	21	21–22
5-Methoxy-4-nitrobenzotriazole	(2)	0·1	14	16–17	20	21 22
4-Amino-5-methoxy benzotriazole	(7)	0·1	16	16–17	17–18	19
2,5-Diamino-4-nitroanisole	(7)	0·1	22	22	..	..
3,4-Diamino-5-nitrophenetole	(2)	0·1	15–16	15–16	16–17	..
2,5-Diacetamido-4-nitroanisole	(7)	0·1	N	N	N	N
4,5-Diacetamido-2-nitroanisole	(2)	0·1	N	N	N	N
4-Amino-2,5-diacetamidoanisole	(7)	0·1	N	N	N	N
5-Aminomethyl-7-hydroxy-I-H-V-triazolo-(d)-pyrimidine	(1)	0·1	N	N	N	N

References to synthesis of compounds:

(1) Unpublished work from this laboratory.
(2) Gillespie *et al.* (1957).
(3) Gillespie *et al.* (1956*a*).
(4) Bost & Towell (1948).
(5) Meldola & Eyre (1902).
(6) King *et al.* (1946).
(7) Gillespie *et al.* (1956*b*).
(8) Fel'dman & Usovskaya (1949).

8) in the early gastrula stage, using the same concentration of 0·1 mg./ml., required only 1–2 hours for 4-M-6-NBD and for 5-NBD, but 3–5 hours for all quinoxalines and for 6-M-4-NBD, and even 10 hours for 5-NBT and 5-NBZ. All other compounds required longer exposures, and even then, after 20 hours,

arrested the embryos at later neurula or tail-bud stages (BD, 4-M-6-NBT, BZ). Sometimes development was normal even in spite of 20 hours' exposure (6-E-4-NBZ, 5-M-4-NBT). To stop all further development and cause death of embryos

TABLE 2

Stage of arrest in development of Rana pipiens *embryos after continuous exposure to a concentration of 0·01 mg./ml. of various chemicals, named according to American Chemical Abstracts nomenclature*

(N = Normal development)

Text code	*Compound*	*Reference to synthesis*	*Embryonic stage (S.) at which development stopped when exposed at*			
			Cleavage S. 3	*Blastula* S. 8	*Neurula* S. 14	*Tail-bud* S. 17/18
BD	2,1,3-Benzothiadiazole	(1)	N	N	N	N
6-M-4-NBD	6-Methoxy-4-nitro-2,1,3-benzothiadiazole	(1)	12	14	22	22
4-M-6-NBD	4-Methoxy-6-nitro-2,1,3-benzothiadiazole	(1)	4–5	12	18	20
5-NBD	5-Nitro-2,1,3-benzothiadiazole	(1)	4–5	10	17	19–20
BZ	Benzimidazole	(1)	N	N	N	N
4-M-6-NBZ	4-Methoxy-6-nitrobenzimidazole	(2)	21	23	N	N
6-E-4-NBZ	6-Ethoxy-4-nitrobenzimidazole	(3)	N	N	N	N
5-NBZ	5-Nitrobenzimidazole	(4)	23	23	23	22
5-M-7-NQ	5-Methoxy-7-nitroquinoxaline	(5)	5–6	15–16	23	23
7-M-5-NQ	7-Methoxy-5-nitroquinoxaline	(6)	5–6	13	23	23
7-E-5-NQ	7-Ethoxy-5-nitroquinoxaline	(3)	5–6	13–15	23	23
6-NQ	6-Nitroquinoxaline	(7)	7	22 24	22–24	22–24
5-M-4-NBT	5-Methoxy-4-nitrobenzotriazole	(3)	N	N	N	N
4-M-6-NBT	4-Methoxy-6-nitrobenzotriazole	(2)	17	17	19	20
6-E-4-NBT	6-Ethoxy-4-nitrobenzotriazole	(3)	12	12–13	18–19	19 20
5-NBT	5-Nitrobenzotriazole	(8)	13	14	16	19

References to synthesis of compounds:

(1) Unpublished work from this laboratory.
(2) Gillespie *et al.* (1954).
(3) Gillespie *et al.* (1957).
(4) Bamberger (1893).
(5) Gillespie *et al.* (1956*a*).
(6) Mizzoni & Spoerri (1945).
(7) Hinsberg (1896).
(8) Hofmann (1860).

first exposed in tail-bud stage 18, using the same concentration of 0·1 mg./ml., required only 5–6 hours for 5-NBT, for 4-M-6-NBD, and for 5-NBD, but it took 12–15 hours for 4-M-6-NBT, and at least 30 hours for 5-NBZ and the quinoxalines. Exposure at stage 18 also showed again clearly that 5-NBZ is the most effective and the unsubstituted BZ the least effective in the benzimidazole series. Thus 24 hours of exposure to BZ permitted almost normal development to stage 24, while 24 hours of exposure to 4-M-6-NBZ and only 8 hours to 5-NBZ already caused arrest at stage 22/23 as abnormal hydrops embryos.

Varying the length of exposure confirmed the observation that the benzotriazole compounds have a greater effect on later embryonic stages and that the nitro-substituted compounds are the most effective inhibitors in each of the groups with the possible exception of the quinoxalines.

Histological examination of the embryos confirmed the morphological obser-

vations, and showed that the benzothiadiazoles, benzimidazoles, and quinoxalines cause similar typical abnormalities and developmental arrests varying only in degree and depending, of course, on stage exposed. In contrast, however, the benzotriazoles cause a different type of arrest and hypomorphism. In the former there is always a selective cellular response, necrosis of early differentiating cells or of highly active mitotic cells, and enlargement and arrest of sensitive cells in prophase stages. In the benzotriazoles, on the other hand, the whole cell population is arrested equally, all nuclei take on a paler stain, and at the same time there is marked delay in development and often there is swelling of the embryo, particularly in the neurula stage. After exposure to benzotriazoles and return to normal medium (10 per cent. Ringer solution) the embryos, unless they die suddenly, continue with delayed normal or hypomorphic development and a tendency toward cyclopia if exposure took place in the blastula stage. Such drastic delay in development, always characteristic of the benzotriazoles, was, however, also found in a benzimidazole, 5-NBZ, where, on the other hand, it was coupled with even more extensive necrosis of sensitive tissues. Exposure to 5-NBZ at stage 18 destroyed not only early differentiating cells, but practically all cells of the nervous system as well. Even the growing posterior end was severely affected; one day after exposure to 5-NBZ the budding tail had become a swollen edematous ball filled with necrotic elements (Plate, fig. A). The chorda cells failed to segregate and to differentiate from the lateral somite cells (Plate, figs. A, B; compare control embryo, fig. C). Later this tail end turned into a wrinkled epidermal projection containing but a few mesenchyme cells. No regeneration of nerve-cord, chorda, or muscle took place. Instead of a head and a tail, only anterior and posterior epidermal knobs developed in which further growth and differentiation ceased. Despite this, and in the absence of a nervous system except for a few regenerating peripheral cells enclosing the necrotic mass (Plate, fig. E) the embryo survived. All of the benzothiadiazoles, benzimidazoles, and quinoxalines brought about a moderate degree of necrosis of sensitive structures; only a few early differentiating cells were so affected while other cells ceased dividing and remained enlarged. The nitro-substituted compound, 5-NBZ, however, was highly necrotic. 5-NBZ also delayed development of the whole embryo. On the other hand, 5-NBT, which produces similar delay in development, exhibits very little necrotic activity. Figs. B, D, and E (Plate) illustrate this remarkable difference. It is difficult to see how such an abnormal embryo as that produced by 5-NBZ (Plate, figs. A and E) could survive at all, whereas embryos exposed to the benzotriazoles which caused the same developmental delay unaccompanied by necrosis so often die suddenly.

DISCUSSION

These experiments demonstrate that the inhibitory effects of certain heterocyclic compounds are influenced by substituents; enhancement of inhibition is

brought about by nitro-substitution in benzothiadiazoles, benzimidazoles, and benzotriazoles. It is of comparative interest that syndactylism, the teratogenic response of the chick embryo to the naturally occurring imidazoles, pilocarpine, and pilocarpidine, appears to be a qualitative function of the parent structure, and that steric configuration affects activity only quantitatively. In the 'trans' configuration (iso) compounds the teratogenic activity is always more intense than in the 'cis' compounds (Landauer, 1956). Similarly, cleavage of the sea-urchin egg is also inhibited more by 'para' compounds than by 'ortho' or 'meta' compounds (Druckrey, Dannenberg, & Schmähl, 1953).

Histologically, none of the benzotriazoles, not even the highly inhibitory 5-nitro compound, elicit selective cellular responses in the embryo as do the benzothiadiazoles, benzimidazoles, and quinoxalines. 5-NBT resembles 5-NBZ in activity except for the lack of selectivity evinced by 5-NBT. While nitro-substitution increases activity and other substitutions decrease it, the type of the response elicited seems to be determined by the nucleus, not by the substituent. The benzotriazoles all cause one characteristic response, while the other series of compounds, benzothiadiazoles, benzimidazoles, and quinoxalines, cause another response which is perhaps best characterized by selective cellular necrosis.

There is some suggestive evidence that the compounds used in this study, particularly the benzimidazoles, could possibly act as purine and pyrimidine antagonists. Studies of the embryological effects of this type of inhibitory agent have been made by Brachet (1946, 1950), Bieber (1954), Liedke, Engelman, & Graff (1954, 1955), Waddington, Feldman, & Perry (1955). Analysis of specific embryogenic responses has shown that some embryogenic processes can be inhibited in varying degree by a number of the agents employed, while other similar agents have no effect. It has also been shown that some agents, azaguanine and benzimidazole, for example, may have inverse effects on different embryos, i.e. azaguanine is highly inhibitory to chick but not to amphibian embryos, while the situation is reversed for benzimidazole.

While the responses of the embryo are often similar, or differ only in degree, and do not illuminate the underlying biochemical processes, it is of interest that one group of compounds, the benzotriazoles, are more or less uniquely different from the other three groups used in this study. It was shown that the former affect neurula and older stages more than younger ones; they may cause mesodermalization of the notochord or cyclopia (Liedke, Engelman, & Graff, 1954, 1955), while the latter cause selective cellular necrosis of early differentiating cells and sensitive structures. Such differences in response are not found with most agents, i.e. Trypan blue (Waddington & Perry, 1956).

It is, of course, possible that this is not a real difference, but only a matter of degree, similar to that described by Waddington, Feldman, & Perry (1955) as the relatively mild effect of ethionine in comparison to the far more drastic inhibition by azaguanine in the chick embryo. They concluded that 'there is

some over-all parallelism between the pattern of sensitivity of the chick embryo to azaguanine and that of the presumed protein synthesis, indicated by the incorporation of methionine-S^{35} and sensitivity to ethionine'. However, no such parallelism was found by them for amphibian embryos with glycine-C^{14} and methionine-S^{35} (Waddington & Sirlin, 1954; Sirlin, 1955; Sirlin & Waddington, 1956). If it were only a matter of degree, one might assume that the embryo could respond first with limitation of metabolism to maintenance without abnormalization, secondly, with mesodermalization and cyclopia indicating disturbance of inductive gradients and processes, and thirdly, with selective necrosis of sensitive regions. Such sensitive regions vary with the stage of development, and are usually composed of highly active cells concerned with morphogenic movements, mitosis, or early differentiation. One could assume then that the benzotriazoles are relatively mild inhibitors which produce only the first and second response, while the other compounds call forth the third or most severe response. This explanation is illustrated in the Plate, figs. A and B. In both cases the embryos are arrested and delayed in development (first response), but in the 5-NBZ embryo (fig. A) the sensitive dorsal structures are already necrotic (third response), while they are still normal in the 5-NBT embryo (fig. B). The only difficulty is that the explanation fits only these two compounds with the highly active nitro group substituents, but not the others, where selective necrosis of sensitive areas occurs (third response) without delay in development (first response). Hence, sensitive structures are affected first with all compounds other than the benzotriazoles, while less sensitive structures still manage to develop more or less normally.

Presumably, all the compounds used in this study could affect nucleic acid metabolism, but the unique activity of the benzotriazoles is somewhat puzzling. Only with the benzotriazoles does swelling occur with lighter pigmentation and loss of cells before death, particularly in neurula arrests, all signs of surface layer disturbances. It is possible, therefore, that the benzotriazoles may exert some action on the surface coat of the embryo (Holtfreter, 1943) like that of surface chelating or polymerizing agents. Such action has been reported by Gustafson & Hörstadius (1955) for some antimetabolites in studies on echinoderm eggs.

SUMMARY

1. Seventeen of 27 structurally related compounds which could act as purine or pyrimidine antagonists inhibited embryonic development to different degrees.

2. Several benzotriazoles were found to exert similar effects on embryos, the intensity varying with substituents. Older, more differentiated, stages are more susceptible to the benzotriazoles than are early cleavage stages. Exposure to the benzotriazoles at the blastula stage results in hypomorphic cyclopian development without necrosis. There is always a marked delay in the rate of development. Swelling and loss of cells is found, particularly in neurula and tail-bud

stages, and usually is followed by death. These findings suggest that the surface coat is interfered with.

3. All active benzothiadiazoles, benzimidazoles, and quinoxalines have the same effect on the embryo; the intensity of the response varying with substitution. Younger, and particularly cleavage, stages are more susceptible, and resistance increases with age of embryo. Exposed blastula stages are frequently arrested as dwarfed tail-buds showing extensive necrosis, particularly of the nervous system. Rarely is there arrest in neurula stages, or if it occurs, these are abortive neurula-exogastrulae with large yolk-plug, no swelling, and larger-sized cells than is normal for this stage. There is always a selective cellular response: early differentiating and very rapidly dividing cells are most sensitive and become necrotic first, while other cells are enlarged and remain arrested.

4. The type of response is determined by the compound, the degree or intensity of response by the substituted groups. It is greatest if only a single nitro group is substituted in the benzothiadiazoles, benzimidazoles, and benzotriazoles, but not in the quinoxalines in which other substitutions seem to have the same degree of effectiveness. On the other hand, some substituents, amino or diphenyl groups for example, decrease the effectiveness of the quinoxalines and benzimidazoles. No direct correspondence between structure and biological function has been discerned.

ACKNOWLEDGEMENTS

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Grateful acknowledgement is made to Mr. Edward R. Hajjar, of the Department of Medical Photography, Francis Delafield Hospital, for preparation of the photomicrographs.

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EXPLANATION OF PLATE

FIGS. A, B, and C are transverse sections through the posterior end of embryos fixed 2 days after start of experiment when controls were at stage 20; figs. A, B: Carnoy, toluidine blue; fig. C: Smith, Harris's haematoxylin.

FIGS. D and E are transverse sections through the head region of embryos fixed 5 days after start of the experiment when controls were at stage 24; Bouin, Harris's haematoxylin. All magnifications × 100.

FIG. A. Embryo 29*b* II F 12, 24 hours in 0·05 mg./ml. of 5-NBZ from stage 17/18, then 10 per cent. Ringer solution. Note necrosis of neural tube and dorsal somite halves and merging of undifferentiated chorda (*Ch*) with ventral somite halves (cf. figs. B, C).

FIG. B. Embryo 46*b* I F 12, 48 hours in 0·05 mg./ml. of 6-E-4-NBT from stage 17/18, then 10 per cent. Ringer solution.

FIG. C. Control embryo (cf. figs. A, B).

FIG. D. Embryo 47*b* I F 12, 48 hours in 0·05 mg./ml. of 7-E-5-NQ from stage 17/18, then 10 per cent. Ringer solution.

FIG. E. Embryo 29*b* III F 12, 24 hours in 0·05 mg./ml. of 5-NBZ from stage 17/18, then Ringer solution. Note regeneration of mesenchyme and a thin peripheral layer of nervous tissue and compare with arrested enlarged cells of embryo 7-E-5-NQ (fig. D).

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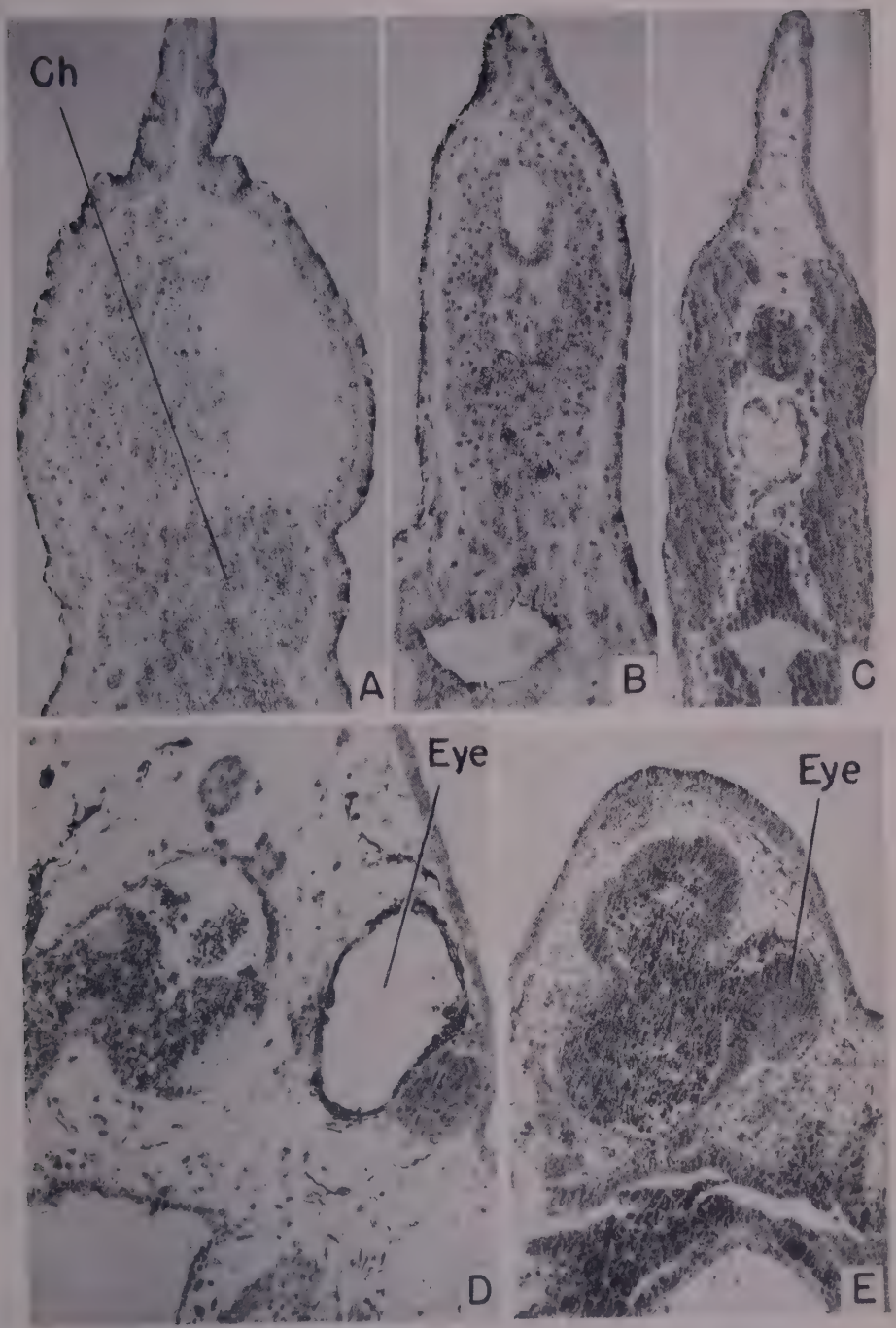

K. B. LIEDKE, M. ENGELMAN, *and* S. GRAFF

Plate

A Comparative Study of the Desoxyribonuclease Activity in Adult and Embryonic Tissue

by GERTRUDE BLUMENTHAL[1]

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INTRODUCTION

RECENT work has pointed to DNA as either the genetic material of the cell or one of its most essential components. As yet the biological role of the desoxyribonucleases (DNases), the only enzymes known to attack polymerized DNA, has not been established. The distribution of these enzymes throughout the animal and plant kingdoms appears to be widespread, and it is now recognized that they fall into at least two general classes (Schmidt, 1955). If these enzymes are involved in chromosome reduplication or in the genetic or developmental controls which may be attributed to nuclear DNA, it might be expected that the pattern of enzyme activity in rapidly growing, undifferentiated tissue and adult differentiated tissues would differ. For these reasons it was decided to investigate the type of DNase activity found in a developing embryonic system as compared to the activity of adult organs of the same species.

The animals studied in this manner were the west coast sea-urchin, *Strongylocentrotus purpuratus*; the frog, *Rana pipiens*; and the domestic fowl, *Gallus domesticus*. These species were chosen since they represented three different types of development according to classic embryological standards. In addition these are embryonic systems where the quantitative changes in DNA and desoxyribonucleotides have been established by previous workers (Hoff-Jorgensen & Zeuthen, 1952; Hoff-Jorgensen, 1954). Any differences in either the level or type of DNase present might then be correlated with both the amount of stored desoxyribonucleotides present and the type of morphogenetic changes which are occurring. It has thus been possible to show a definite difference between adult and early embryonic tissue for all three species studied, and also a difference in the embryonic pattern of DNase which may be associated with the level of stored desoxyribonucleotides in the developing ova.

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MATERIAL AND METHODS

All material to be assayed for DNase activity was homogenized immediately after collection or frozen and stored at − 15° C. Homogenization was carried out in a Potter homogenizer, with the exception of the eggs and embryos of the sea-urchin. These were homogenized in a 'Microblender' consisting of a 15 ml. glass vial containing a set of small brass blades which were electrically driven at about 5,000 r.p.m.

Strongylocentrotus purpuratus

The eggs and sperm of the sea-urchin were collected and fertilization carried out in the manner described by Mazia (1949). Unless the eggs showed 90–100 per cent. fertilization they were not used. This criterion was also applied when only non-fertilized eggs were collected. Mass cultures were grown in an aerated 5-l. Ehrlenmeyer flask rotated at about 20 r.p.m. in a room maintained at 14° C. Quantities of the embryo suspension containing between six and ten million eggs were transferred to 50-ml. centrifuge tubes. The eggs were concentrated by low-speed centrifugation, and almost all the debris and micro-organisms present could be removed with the supernatant sea-water. Repeated centrifuging, utilizing only two centrifuge tubes, was employed in order to minimize any loss which might occur in transferring the packed embryos to the 'Microblender'.

Rana pipiens

The method of Rugh (1934) was used to obtain the eggs and embryos of this species. Only those clutches which showed less than 10–15 per cent. abnormal development were utilized for DNase determinations. At the desired stage of development 100 embryos were removed from the culture dishes and frozen and stored at − 15° C. Eggs and embryos from a single clutch of eggs were used for each set of determinations. Before homogenization the embryos were only partially thawed, so that they could be transferred to the glass vessel of the homogenizer.

Gallus domesticus

Fertilized hens' eggs were obtained from the Division of Poultry Husbandry of the University of California at Berkeley. The eggs were refrigerated after collection, and placed in a 39° C. incubator when it was desired to initiate further development. Eggs were removed at appropriate intervals, the embryos dissected out, and examined microscopically to make certain they were at the developmental stage desired. The embryos were then washed twice with 0·85 per cent. saline to remove any adhering yolk, and homogenized in the manner previously described.

DNase determinations

High polymer DNA was prepared from salmon testes by the method of Bernstein (1953). This procedure yielded a preparation which was white, fibrous, hygroscopic, and dissolved readily in water to give a clear viscous solution.

The substrate normally employed contained 0·15 per cent. DNA, and was either 0·024 molar with respect to magnesium chloride, or 0·03 molar with respect to sodium citrate. When the substrate was buffered at a pH below 6·0, a 0·1 molar acetate buffer was used. For those determinations carried out at a pH above 6·0, a 0·2 molar phosphate buffer was used.

The reaction mixture used for the determinations of DNase activity in the sea-urchin eggs and embryos consisted of 10 ml. of the substrate solution and 1·0 ml. of the enzyme sample. The enzyme activity of this material was such that 1·0 ml. of homogenate containing from 300,000 to 500,000 eggs or embryos was sufficient to measure the depolymerization of 15 mg. of DNA, and the enzyme activity was proportional to the quantity of embryonic material in the homogenate. For the analysis of adult tissues of this species, 1·0 ml. of homogenate containing from 2·0 to 6·0 mg. of protein fulfilled the requirements that the DNase activity should be proportional to the amount of enzyme used.

For most of the determinations the protamine precipitation method of Barton (1948) was used. The homogenate and buffered substrate were incubated with shaking at 27° C. At zero time (i.e. upon addition of the homogenate to substrate), and at desired intervals during the course of the reaction, the amount of protamine-soluble DNA present in a 2·0-ml. sample was determined spectrophotometrically.

The amount of DNA liberated in low polymer form for any period during the course of the reaction was calculated as the difference between the zero time value and the value after the given period of incubation. For a comparison of the activity at different stages of development, the activity was expressed as milligrammes of low polymer DNA liberated per hour per embryo. When it was desired to make a comparison of the activity in embryonic and adult tissues, or between two types of adult tissues, the activity was expressed as milligrammes of low polymer DNA liberated per hour per milligramme of protein of the homogenate. The rate of depolymerization was determined for each measurement, and the calculation of activity was made from values obtained from that portion of the curve which was linear.

Viscometric determinations

Although the viscometric method of DNase assay may be subject to a number of limitations, it can be used to detect small amounts of activity which might not be revealed by the other methods in use. When the protamine precipitation method revealed no activity in the embryonic stages of the frog and chick, the viscometric method was used to verify the results.

The procedure used for the viscosity measurements was similar to that of Laskowski & Seidell (1945). Measurements were made with an Ostwald viscometer at 27° C. The substrate was a 0·11 per cent. solution of DNA which was 0·024 molar with respect to magnesium, or 0·03 molar with respect to citrate. It was buffered with 0·2 molar phosphate for determinations at pH 6·0 or above, and with 0·1 molar acetate for determinations below 6·0. Both the homogenate and substrate were allowed to attain a temperature of 27° C. before being added to the viscometer. Five millilitres of substrate and 3 ml. of homogenate containing 0·4 to 1·3 mg. of protein per millilitre were used for each determination. A measurement was made immediately after mixing the homogenate and substrate and at approximately 5-minute intervals thereafter. When the reaction had proceeded for over 30 minutes, measurements were taken at less frequent intervals until the viscosity had attained a constant value.

For certain stages of the chick embryo, where there seemed to be an increase of activity as development proceeded, reaction velocity constants were calculated. These were calculated from the formula $K = 1/t \log(n_0/n_1)$ in which n_0 is the relative viscosity at zero time and n_1 is the relative viscosity at time t.

Protein determination

The concentration of protein in the homogenates was determined according to the method of Lowry *et al.* (1951). A calibration curve for known concentrations of crystalline bovine albumin was used as the standard.

RESULTS

Embryonic tissues

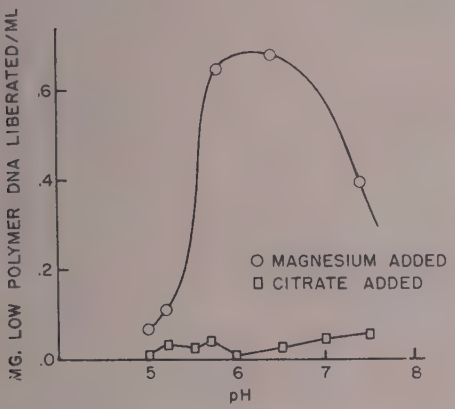
Of the three species studied, only the sea-urchin exhibited any appreciable activity during the early stages of embryogenesis. Not only was the amount of DNase activity per embryo constant throughout development, but also the characteristics of the enzyme showed no changes. Those stages investigated included unfertilized eggs, fertilized eggs just prior to the first cleavage, 4- and 16-cell stages, early blastulae, free-swimming blastulae, gastrulae prisms, and plutei.

Homogenates were tested for activity in the pH range of 5–7 with reaction mixtures containing magnesium and those from which the magnesium had been removed by the addition of citrate. All stages tested showed no activity when magnesium was absent from the reaction mixture. In Text-fig. 1 it can be seen that the pH optimum for the DNase activity of unfertilized egg is between 6·6 and 6·8. The pH activity curves for the other stages are essentially the same.

The fact that this DNase activity is magnesium activated and has a pH optimum of about 6·8 places it in the DNase I category (Cunningham & Laskowski, 1953).

Table 1 shows the amount of activity of this magnesium-activated enzyme

which is present in the eggs and embryos. The difference in activity of the various stages is of the same order of magnitude as the difference obtained between different determinations on the same stage of development. The values given here are the mean values for from 2 to 4 determinations on different cul-



TEXT-FIG. 1. pH optimum of egg homogenates.

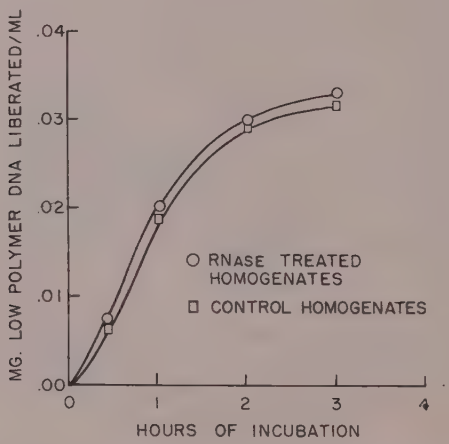
TABLE 1

DNase activity of eggs and embryos, expressed as mg. $\times 10^{-7}$ of low polymer DNA liberated per hour per egg or embryo

Developmental stage	*Age*	*DNase activity*
Unfertilized egg	0	2·8
Fertilized egg	50 minutes	3·0
2-cell	2 hours	2·9
16-cell	3·5 hours	2·1
Early blastula	14 hours	2·7
Blastula	17 hours	2·8
Gastrula	31 hours	2·0
Late prism	68 hours	2·7

tures of embryos. These data are in agreement with those obtained for Arbacia (Mazia, 1949), where it was also found that the level of activity remained constant throughout development. Although no difference in developmental pattern of the enzyme was expected between such closely related species, it was felt that an analysis at more frequent intervals after fertilization might show changes in activity not indicated by the study on Arbacia. However, the results obtained from these determinations do not indicate any changes in the level of activity which might be correlated with mitotic activity or changes in DNA metabolism.

Recent work has shown that in many forms an RNA inhibitor is present, which masks the true level of DNase activity of the organism (Roth, 1954; Kozloff, 1953 *a*, *b*). It seemed possible that such an inhibitor might regulate differences in the *in vivo* level of DNase activity during development and that these differences would not be detectable by the methods used. The DNase of Arbacia is in the soluble portion of the homogenate of unfertilized eggs and, as development proceeds, becomes progressively localized on the sedimentable



TEXT-FIG. 2. Activity of RNase-treated plutei homogenates.

portion. This appearance of the DNase of later stages in a sedimentable form suggested that it becomes associated with the particulate matter of the cytoplasm, which is rich in RNA. Such a close association with cytoplasmic RNA might mask a potentially higher activity at later stages of development. For these reasons it seemed worthwhile to study the effect of RNase pretreatment of the homogenates on the level of activity of the various developmental stages.

Homogenates were incubated for 30 minutes at 27° C. with a 0·2 per cent. solution of crystalline RNase (Worthington Biochemical Corporation), and buffered at pH 6·0 before they were added to the substrate. The activity of these homogenates was compared to those of untreated homogenates of equal concentration. Text-fig. 2 shows representative results of one of such a series of determinations made on the different developmental stages. It is apparent that there is no significant difference in activity between the RNase digested and the untreated homogenates. At present there is no evidence to support any contention that the constancy of DNase activity throughout the embryonic development of this species is an artifact of the method of assay.

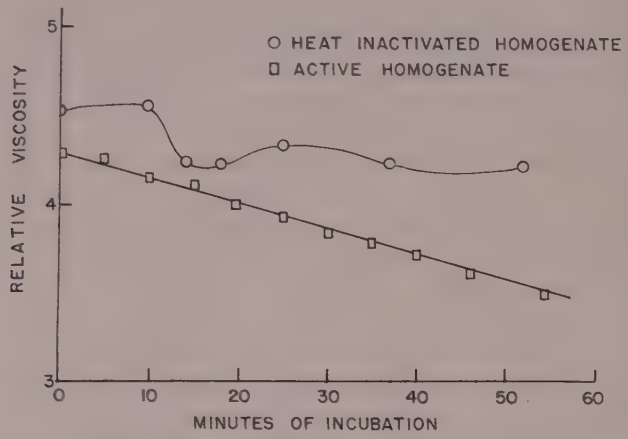
Unlike the sea-urchin, the eggs and embryos of the frog exhibited no DNase activity. Using the protamine precipitation method, no appreciable activity of an enzyme corresponding to DNase I or II could be detected in any of the embryonic stages from unfertilized eggs to free-swimming tadpoles. With the viscometric method of DNase assay, which is more sensitive than the protamine method, no activity of either acid or neutral DNase could be detected in stages up to and including Shumway stage 25 (Shumway, 1940). Here, too, homogenates were tested for activity in the pH range of from 4 to 7, both in the presence and absence of magnesium. Although not amenable to quantitative interpretation, the viscometric method is the most sensitive method now available for detecting DNase activity. The complete absence of these enzymes cannot be proved conclusively, but it seems reasonable to assume that they are not present in any functionally significant amounts unless they can be detected by this method. Pretreatment of the homogenates by methods known to destroy the usual inhibitors of DNase did not result in any active enzyme preparations. This included digestion of the homogenate with RNase and the dilute acid treatment described by Cunningham & Laskowski (1953) for the removal of inhibitors from calf-kidney preparations.

It is somewhat difficult to explain the discrepancy between these results and those obtained by Finamore (1955). He reports changes in the level of DNase activity during the early development of *Rana pipiens*. The increase in ultra-violet absorption which he obtained for those stages which had maximum activity was rather low, especially when compared to the values for RNase activity in the same material. Since he did not indicate if measurements had been made of the amount of ultra-violet absorbing material released by incubation of the homogenates alone, there is no way of distinguishing what proportion of the optical density was actually due to the products released by DNase action. Even if one accepts Finamore's data as given, the difference between his results and those of this investigation becomes merely a question of whether there is no DNase activity or extremely low activity in the early developmental stages of the frog.

Allfrey & Mirsky (1952) had previously demonstrated the presence of DNase II activity in the organs of the 15-day chick embryo, the 8-day chick, and adult chickens. They did not report the presence of any DNase I in this material. Their work did not indicate whether this enzyme was present throughout development or appeared only at later stages. The determination of DNase activity in early stages of chick embryogenesis was undertaken in order to answer this question and also to determine whether DNase I might be present in earlier stages.

When the protamine method was used, assays for DNase I and II did not show any activity in the 24-, 48-, 72-, and 96-hour chick embryo and in yolk isolated from 24-hour embryos. Viscometric assays also did not reveal any activity in stages prior to the 96-hour chick. At this stage of development a small amount of DNase II could be detected. In Text-fig. 3 it can be seen that the DNase

activity at this stage is only just within the range of detectability even when viscometric methods are used.



TEXT-FIG. 3. DNase I activity of 96-hour chick embryo.

TABLE 2

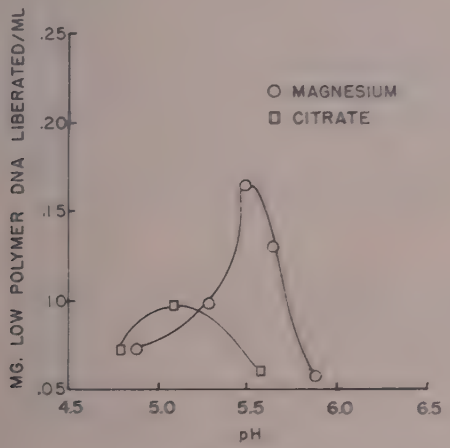
DNase II activity of pre-hatching Chick, expressed as the velocity constant

Age in days of incubation	*DNase II activity*
4	$0{\cdot}17\times10^{-2}$
6	$0{\cdot}87\times10^{-2}$
7	$1{\cdot}65\times10^{-2}$
8	$1{\cdot}73\times10^{-2}$
10	$2{\cdot}73\times10^{-2}$

No activity of the DNase I type could be detected at this stage of development or later when the activity of the DNase II increased.

The progressive increase of DNase II from the 4th to the 10th day of incubation is shown in Table 2. This activity is expressed as the velocity constant per amount of embryo in the added homogenate. For all of these determinations the protein concentration of the added homogenate was in the range of 1·0 to 2·0 mg./ml. This was achieved by decreasing the number of embryos homogenized and increasing the total volume of homogenate as the age of the embryo increased. The advantage of this procedure over that of keeping the number of embryos homogenized constant for each stage tested was that it avoided the possibility of spurious differences in the rate of viscosity decrease between homogenates of different embryonic stages due to differences in total protein content.

The velocity constants were calculated from several measurements taken during the period of linear viscosity decrease. The values given in Table 2 are the mean of from 2 to 4 determinations on different homogenates. The deviation from the mean for the activity values calculated in this manner was never greater than $0{\cdot}15 \times 10^{-2}$. It is obvious that the amount of enzyme per embryo increases progressively after the 4th day of incubation; although the percentage increase cannot be calculated with any degree of accuracy. The presence of any DNase II prior to 96 hours of incubation seems improbable, since more embryonic material was present in the homogenates of early stages than in those where activity was demonstrable. This is due to the fact that all homogenates assayed had equivalent protein concentrations.



TEXT-FIG. 4. pH optimum of adult gut homogenates.

Adult tissues

Analysis of the only two tissues of the adult sea-urchin which could be removed in an intact state, gut and ovary, revealed the presence of a DNase whose properties differed from that found in the eggs and embryos of this species. The activity is only partially inhibited by concentrations of citrate which completely inhibit embryonic DNase. In addition there is essentially no activity at the pH where the embryonic enzyme exhibits maximum activity. Text-fig. 4 shows the pH optimum and the effect of citrate on this adult enzyme in gut homogenates. The effect of various inhibitors and activators of crystalline DNase on the DNase activity of this homogenate is shown in Table 3.

Those compounds which remove magnesium from the medium, such as citrate and versene, only reduce the activity by less than one-half, whereas the same agents completely inhibit DNase I. Manganese, which activates most

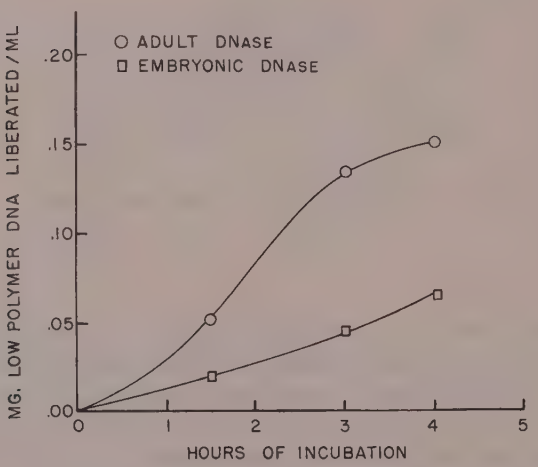
DNase I preparations to practically the same extent as magnesium, inhibits this activity to an even greater extent than does fluoride.

TABLE 3

Effect of inhibitors and activators on adult DNase. Enzyme activity is expressed as milligrammes per millilitre of low polymer DNA liberated per hour

Substances used and concentration in final reaction mixture	*Enzyme activity*
0·02 molar magnesium chloride . . .	(1)* 0·114
	(2)* 0·115
0·02 molar manganese chloride . . .	(1) 0·058
	(2) 0·045
0·02 molar sodium fluoride	(1) 0·108
	(2) 0·096
0·025 molar sodium citrate	(1) 0·043
	(2) 0·032
1×10^{-3} molar versene	(1) 0·042
	(2) 0·037

* (1) and (2) designate different experiments performed with homogenates containing the same concentration of protein.



TEXT-FIG. 5. DNase activity of ovarian homogenates.

The shifting of the pH optimum from 5·1 to 5·5 in the presence of magnesium and the fact that this enzyme is active in the absence of magnesium both tend to suggest that the apparent magnesium activation may not be due to any effect on the enzyme itself. The activation is probably due to the effect of the magne-

sium on the substrate. Tamm, Shapiro, & Chargaff (1952) have shown that magnesium will cause a further degradation or depolymerization of partially digested DNA. It is probable that this enzyme of adult tissues only partially depolymerizes the substrate, and in the presence of magnesium it is further degraded.

The fact that magnesium does not inhibit adult DNase activity keeps this enzyme from rigidly fulfilling the DNase II qualifications. However, its characteristics more closely resemble this type of activity than those of DNase I. On the basis of the characterization of the two types of DNase found in mammalian tissues, it would seem valid to conclude that the DNase found in the gut and ovaries of the adult sea-urchin is due to an enzyme which is separate and distinct from that found in the eggs and embryos. In the gut homogenates no activity was found which was similar to that found in the embryos.

In homogenates of ovaries from which practically all the mature eggs had been shed and only immature oocytes remained, both types of activity were present. Text-fig. 5 shows the results of determinations for both types of activity in the same ovarian homogenates. Although embryonic DNase is present, the adult type predominated.

TABLE 4

Comparative activity of different tissues of Strongylocentrotus purpuratus

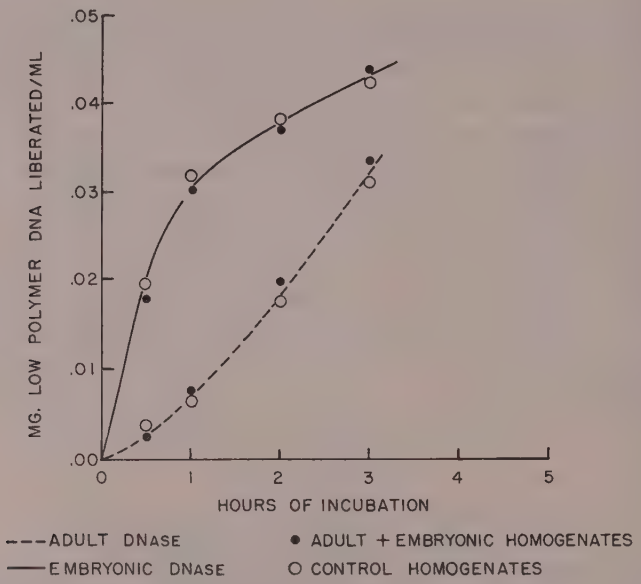
Activity is expressed as mg. low polymer DNA liberated per hour per mg. of protein in the homogenate and the values are averages of at least 3 separate determinations whose mean deviation was never greater than $0{\cdot}5\times10^{-2}$

Tissue	*Activity of embryonic DNase*	*Activity of adult DNase*
Gut	$0{\cdot}0\times10^{-3}$	$6{\cdot}2\times10^{-2}$
Ovary	$6{\cdot}2\times10^{-3}$	$1{\cdot}8\times10^{-3}$
Blastulae	$4{\cdot}5\times10^{-3}$	$0{\cdot}0\times10^{-3}$
Unfertilized eggs	$4{\cdot}5\times10^{-3}$	$0{\cdot}0\times10^{-3}$

The data presented in Table 4 show that the amount of activity of both enzymes, calculated on the basis of the protein content of the tissues, is lower in the ovary than in those tissues where only one type of activity is present. The embryonic DNase is only 13 per cent. of that found in mature fertilized eggs. It seems probable that the embryonic DNase activity in this tissue is due to the presence of oocytes and the few mature eggs which might still remain in the ovary.

Unless inhibitors are present it can be concluded that embryonic cells contain only one type of DNase, while differentiated adult cells exhibit only activity of another type. The fact that the ovary exhibited both types could exclude the

possibility of the presence of inhibitors. However, the low levels of activity found in this tissue might be explained by partial inhibition of both enzymes. To test the possibility of inhibitors masking the presence of adult DNase in embryonic tissue and embryonic DNase in differentiated cells, homogenates of the



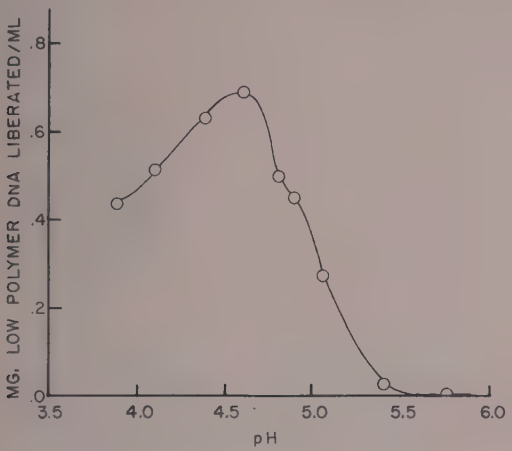
TEXT-FIG. 6. Absence of inhibitors in adult and embryonic tissues.

gut were mixed with homogenates of unfertilized eggs or embryos and both types of activity determined. These activities were compared to that of adult and embryonic tissue alone. Text-fig. 6 shows the results of such an experiment. It is obvious that neither tissue exerts an inhibitory effect.

From these experiments it can be concluded that the adult sea-urchin contains a DNase which is not present in either the eggs or any of the developmental stages up to and including the pluteus larva. It is probable that this enzyme first makes its appearance sometime during the metamorphosis of the pluteus larva to an adult sea-urchin. The absence or extremely low level of the embryonic enzyme in adult tissue suggests that during or after metamorphosis of the pluteus larva this enzyme is either destroyed or converted to another type.

Homogenates of various adult organs of the frog exhibited DNase activity in the pH range of 4 to 5. Although magnesium did not completely inhibit this activity, it was higher in reaction mixtures from which the magnesium had been removed by the addition of citrate. Text-fig. 7 shows the pH optimum for the DNase activity found in homogenates of adult frog-liver. The optimum was

essentially the same for other adult tissues tested. Above pH 6 no appreciable activity could be detected either in the presence or absence of magnesium. The effect of the addition to the reaction mixture of some of the usual inhibitors and



TEXT-FIG. 7. pH optimum for DNase of adult frog-liver.

activators is shown in Table 5. The source of the enzyme tested here was a homogenate of adult stomach mucosa. The activity of the enzyme was inhibited by the presence of magnesium or those substances which did not completely remove magnesium from the medium. The enzyme activity found in these adult frog-tissues shows the same characteristics as that described for DNase II in bovine tissues.

TABLE 5

Effect of inhibitors and activators on frog DNase II. Enzyme activity is expressed as milligrammes per millilitre of low polymer DNA liberated per hour per mg. of protein of homogenate

Substance used and concentration in final reaction mixture	*Enzyme activity*
0·02 molar magnesium chloride . . .	(1)* $2{\cdot}2\times10^{-2}$
	(2)* $2{\cdot}2\times10^{-2}$
0·025 molar sodium citrate	(1) $3{\cdot}4\times10^{-2}$
	(2) $3{\cdot}4\times10^{-2}$
1×10^{-3} molar versene	(1) $2{\cdot}6\times10^{-2}$
	(2) $2{\cdot}4\times10^{-2}$
Equal volume of double distilled water .	(1) $3{\cdot}0\times10^{-2}$
	(2) $2{\cdot}8\times10^{-2}$

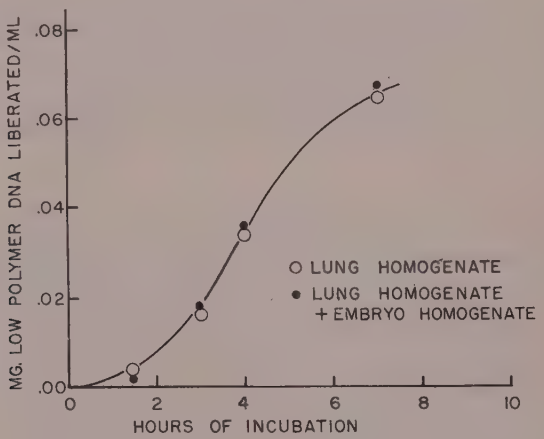
* (1) and (2) designate different experiments performed with homogenates of equal concentration.

TABLE 6

DNase activity of adult frog-tissues, expressed as milligrammes per millilitre of low polymer DNA liberated per hour per mg. of protein of homogenate. The values listed are each the average of three determinations whose mean deviation was never larger than $0{\cdot}10 \times 10^{-2}$

Tissue	*DNase activity*
Stomach . . .	$2{\cdot}04 \times 10^{-2}$
Lung	$0{\cdot}94 \times 10^{-2}$
Ovary	$1{\cdot}47 \times 10^{-2}$
Kidney . . .	$2{\cdot}90 \times 10^{-2}$
Liver	$4{\cdot}05 \times 10^{-2}$

The relative amounts of this DNase II in various adult frog-tissues is shown in Table 6. The distribution of activity in the adult frog is similar to that obtained for several mammals by Allfrey & Mirsky (1952). Although ovarian eggs showed no DNase II activity, the activity found in ovaries collected after the breeding season is higher than that found in an organ such as the lung. Most probably this enzyme is present only in the somatic cells of the ovary. Therefore, the level of



TEXT-FIG. 8. Effect of embryonic tissues on adult DNase.

activity in the actual ovarian tissue might be of the same order of magnitude as that of the liver or kidney. The possibility still existed that the presence of an inhibitor in embryonic tissue might be masking the presence of DNase II in the eggs and embryos. This was ruled out by testing the effects of embryonic tissues on the activity of adult lung homogenates. This tissue was chosen, since its low

level of activity should make it an exceedingly sensitive test system for the presence of inhibitors. Lung homogenates were mixed with homogenates of unfertilized eggs or early cleavage stages and the rate of depolymerization measured. Text-fig. 8 shows the results of such a determination.

The absence of DNase II in the eggs and embryos of the frog and its presence in every adult organ tested suggested that it first made its appearance during metamorphosis. To determine during which period of metamorphosis this occurred, tadpoles were collected at different intervals after hatching, homogenized, and tested for DNase activity by the viscometric method. The first appreciable amount of DNase that could be detected was in tadpoles that had been raised for 50 days after they attained Shumway Stage 25. These tadpoles, measured from the head to the tip of the tail, ranged from 40 to 50 mm. in length. They had completely formed eyes. Limb-buds were present, but none of the limbs had yet emerged. A homogenate of two such embryos, having a protein concentration of 1·2 mg./ml., depolymerized the substrate during the first 18 minutes of the reaction with a velocity constant equal to $1{\cdot}88 \times 10^{-2}$. An adult frog-kidney homogenate having a protein concentration of 0·50 mg./ml. and acting on the same concentration of substrate, had a velocity constant of $2{\cdot}73 \times 10^{-2}$. From even this sort of crude comparison it is obvious that the DNase activity increases to a large extent from the time it makes its first appearance until the frog reaches maturity.

DISCUSSION

Of the three species studied, only the sea-urchin exhibited any DNase activity during the early portion of embryogenesis, when most of the cell-division and differentiation occurs. There seems to be little doubt that this activity is due to an enzyme which is separate and distinct from that found in the other two species and the adult tissues of the sea-urchin. The disappearance of this enzyme in the adult would tend to support the contention that this enzyme probably has an essential role in either the maintenance, division, or differentiation of embryonic cells. The fact that the amount of enzyme does not change during development, even at periods when cell-division and differentiation are at their maximal level, neither supports nor disproves this contention. However, the question then arises as to why this enzyme or any DNase cannot be found in equivalent embryonic stages of the chick or frog.

There appears to be a fundamental difference in chemical composition of the egg of the sea-urchin as compared to the egg of the frog or chick, which might explain this difference in DNase activity. The quantity of yolk and yolk-like material is much greater in the eggs of the latter two species than in that of the sea-urchin, and the amount of stored desoxyribonucleotides seems to parallel the quantity of yolk present. Therefore, the presence or absence of DNase in early embryonic stages might well be dependent on the amount of *de novo* DNA

synthesis required of the developing embryo. Hoff-Jorgensen has shown that the sea-urchin contains only enough preformed DNA or desoxyribonucleotides to carry it to the 16-cell stage, while the frog has enough for development to late blastulae or a 5,000-cell stage, and the chick enough for the formation of 5×10^7 cells.

The distribution of DNase II and the period of development at which it first appears is essentially similar for all three species studied. The increase of activity of this enzyme during late development, as well as the period when it first emerges, establishes its appearance as a result of differentiation or maturity rather than as one of their causes. In both the frog and the sea-urchin the enzyme first makes its appearance during metamorphosis. In the chick it appears on the 4th day of incubation, and the chick at this time cannot be considered to be any less advanced embryogenically than a metamorphosing sea-urchin or frog tadpole. The heart has already differentiated into its component parts, the digestive tract has become subdivided, the pancreas and internal mesenteries have been formed, and most of the structures of the adult eye are present.

Although there is a clear association of DNase II with adult differentiated tissue, no one has set forth a satisfactory explanation of how this enzyme is involved in either the growth or maintenance of these adult tissues and organs. Allfrey & Mirsky (1952) correlate the amount of enzymes present in adult tissues with the tissue's ability to proliferate or regenerate. On the basis of this observation and the rate of glycine-N^{15} incorporation into the nuclear DNA of these tissues, they postulate that DNase II is related to the overall desoxyribonucleotide metabolism, which is higher in those tissues capable of proliferation or regeneration. The role which Allfrey & Mirsky postulate for the function of DNase II does not account for its absence in embryonic tissue or its low activity in differentiated organs during periods of most rapid growth.

From the data obtained by the authors mentioned above, the results of this study, and the work of Brody (1953) it seems quite possible that this enzyme may be more closely associated with the destruction and removal of dead or non-functional cells than with the synthesis or overall desoxyribonucleotide metabolism of the tissues in question. The amount of this enzyme in any specific organ seems to increase with the age of the organ (Allfrey & Mirsky, 1952). The degree of destruction or degeneration of the cells of many organs and tissues may also increase with age. This is exemplified by the decrease in the number of cells and fibres in the ganglia and spinal nerves of man (Gardner, 1940); the loss of glomeruli in rat kidney (Aratki, 1926); and the decrease in epidermal cell-layers of the human head and face with advancing age (Ejiri, 1937). The high enzyme activity of mammalian spleen from many sources and the blood-destroying function of this organ also tends to support such a hypothesis. The spleen has from 2 to 5 times the activity of the liver and intestinal mucosa which are among the richest sources of this enzyme (Allfrey & Mirsky, 1952).

Brody (1953) has found that the DNase activity of the 10-weeks' old human

placenta is approximately ten times greater than that of a full-term placenta. The amount of DNase, calculated with the DNA content of the tissue as a basis of reference, remains constant during the 10th and 12th weeks of pregnancy and then progressively diminishes, reaching the minimum value at the 40th week. This decrease in DNase activity cannot be attributed to a cessation of growth, since the area of the uterus which the placenta occupies increases by about 16 per cent. from the 10th to the 20th week, while the enzyme activity drops by more than one-half. However, it is during these first 10 weeks of pregnancy that the formation and growth of the placenta is dependent upon invasion and cell destruction as well as cellular growth and division. Since the cytolytic processes accompanying placental growth, such as invasion of the endometrium and the disappearance of the chorionic villi in the region of the decidua capsularis, all come to a halt at about the 10th week of pregnancy, this too can serve as an example of a relationship existing between the level of DNase activity and the amount of cell destruction occurring in a given tissue or organ.

The absence of DNase II in the early embryo might also be explained in part by the amount of cell death and degeneration associated with the embryonic development of these species. Metamorphosis in the sea-urchin results in such drastic changes in both the organ systems present and the overall appearance of the animal that it is difficult to imagine this occurring without a relatively large degree of degeneration or removal of many of the original larval structures. Though there may be cell death and degeneration occurring during development prior to metamorphosis, there is little or no evidence for this in the literature.

In the case of the frog and chick there is evidence of cell death and degeneration in embryonic stages prior to those at which DNase II first makes its appearance. Glucksman's review (1951) lists the incidence and localization of cell degenerations during normal vertebrate ontogeny. The first instances of cell destruction cited for *Rana* is that of degeneration in ganglia of branchial nerves during metamorphosis. It is possible that embryonic cell degeneration does occur earlier than this, as Glucksman lists the degeneration of yolk endoderm cells during gastrulation of another amphibian. However, the paucity of such information in the literature does suggest that the greatest degree of such degeneration occurs during metamorphosis, the period of development when this enzyme activity could first be detected. There are numerous observations of histolysis and cell degeneration in the developing chick embryo prior to the 4th day of incubation (Glucksman, 1951). Though there appears to be more sites of cell degeneration during subsequent development of the chick embryo, it is impossible to state definitively that this process is more pronounced in later stages where the DNase II content increases.

Leblond & Walker (1956) have recently focused attention on the extent of cell destruction and renewal which occurs in the animal body. Their work indicates a much lower order of magnitude for the turnover time of many cell populations than hitherto accepted. Destructive enzymes such as DNase II may be of the

highest significance in the maintenance of these renewal processes and the normal economy of the animal body.

SUMMARY

1. Determinations have been made of the desoxyribonuclease (DNase) activity of various embryonic stages and adult tissues of a sea-urchin, a frog, and the domestic fowl. Special attention has been paid to the pH optimum and the magnesium requirement of the enzymes. Of the three species investigated only the sea-urchin exhibited DNase activity during early embryonic development. This activity was essentially the same as that of crystalline bovine pancreatic DNase, although it is doubtful that the two enzymes are identical. The possible correlation of this enzyme with the amount of preformed desoxyribonucleotides in the egg is discussed.

2. At later stages of development, all three species contain an enzyme which is similar to DNase II of mammalian origin. The possibility that the difference in enzyme activity between embryonic and mature tissue is due to the presence of inhibitors has been ruled out. The data obtained in this study and that of other workers suggests that the adult DNase may play a role in the processes of cell destruction and cell turnover in adult metazoans.

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Embryological Development of Primordial Germ-cells in the Mouse: Influence of a New Mutation, W^{j}[1]

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WITH TWO PLATES

INTRODUCTION

THE pleiotropic mutant genes W and W^{v} are alleles of w in the mouse, and produce anaemia, absence of fur pigmentation, and sterility in homozygotes (review by Russell, 1954). Germ-cells of both male and female homozygotes are lacking or drastically reduced in numbers at birth, the genotypes being identifiable through the concurrent anaemia. The developmental basis for this sterility was therefore sought in embryonic life and has been described (Mintz & Russell, 1955, 1957).

Recently, a new mutation, W^{j}, with comparable effects in the homozygote, arose at the same locus. Evidence that it is an allele of the W-series, but diffferent from W or W^{v}, will be presented elsewhere (Russell, Lawson, & Schabtach, *in preparation*).

In the present report, the early abnormalities characterizing $W^{j}W^{j}$ will be traced and compared with those produced by the other mutant alleles, and will be considered in relation to the problems of germ-cell origin and pleiotropism. A preliminary note (Mintz, 1957) has appeared on the study.

Initial appearance of the primordial germ-cells in the mouse has been noted (Chiquoine, 1954; Mintz & Russell, 1955) at 8 days, when they are seen in the yolk sac splanchnopleure, caudal end of the primitive streak, and root of the allantois (Plate 1, fig. A). Migration through neighbouring tissues begins at 9 days, when the cells occur in the gut splanchnopleure and may proceed up the dorsal mesentery of the gut (Plate 1, fig. B). At 10 days they are found in the mesentery and at its root, near the dorsal aorta, around the coelomic angles (Plate 2, fig. D), in the mesonephric regions, and in the paired germinal ridges.

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(A detailed description of this migratory route, with excellent illustrations, was presented by Witschi in 1948 for the human embryo, in which the germ-cells are large and readily recognizable with routine histological stains.) Migration is largely completed at 12 days in the mouse. During this 4-day period the germ-cells continue to multiply, from a maximum of 76 at 8 days to a maximum of 5,711 at 12 days, in *ww* controls (Mintz & Russell, 1957).

The later gonad sterility in W^iW^i could conceivably be due to non-formation of primordial germ-cells, excessive mortality after formation, mitotic failure, retarded migration or non-migration to the gonads, migration to ectopic sites, or a combination of these. In the case of W^iW^i, as with WW, W^vW^v and WW^v, the defect proved to be a lack of increase in total numbers; this was coupled with retarded migration of surviving cells.

The techniques first adopted for the study of this type of problem were described in full in the previous account concerning WW, W^vW^v, and WW^v embryos (Mintz & Russell, 1957) and will be summarized only briefly here.

MATERIALS AND METHODS

Non-viability and sterility of W^iW^i individuals made it necessary to depend on matings between the fertile W^iw heterozygotes to obtain defective embryos. These would be expected to occur with a frequency of 25 per cent. and are not phenotypically identifiable by other defects at the ages investigated.

Since the mutation occurred in the C3H inbred strain, the animals used were isogenic except for W^i segregation. Twenty-seven embryos were preserved, one litter at 8 days of age, one at 9 days, and one at $10\frac{1}{6}$ days. This range of time was selected because observations on the other alleles showed their effects on germ-cells to be first visible at 9 days; this was confirmed for W^iW^i. Eight-day embryos were fixed *in utero*; older ones were removed and dissected free from membranes.

Because of the difficulty of identifying primordial germ-cells in the mouse by means of conventional stains, and the uncertainty which would therefore preclude an exact quantitative survey, a histochemical procedure for alkaline phosphatase was employed. Conspicuous elective staining of these cells because of their high alkaline phosphatase content has been reported with both the Gomori calcium-cobalt method (Chiquoine, 1954, mouse; McAlpine, 1955, rat) and the azo dye coupling procedure (McKay, Hertig, Adams, & Danziger, 1953, human). The Gomori (1951) modification of the latter technique, employing sodium alpha-naphthyl phosphate as substrate, was found to yield excellent results. Nuclear staining is absent; reacting cytoplasm is bright red (with the diazonium salt used). The identity of the few other phosphatase-positive tissues is known.

In connexion with the earlier study, a number of detailed projection drawings were made (Mintz, unpublished data) of sections stained for alkaline phosphatase, in which every germ-cell was outlined. Cover slips were then removed, the

azo dye product washed out in alcohol, the tissues re-stained in Delafield's haematoxylin and eosin, and the sections re-projected on to the drawing. In a cell-for-cell comparison, identical cells were detectable as germ-cells with both staining techniques. However, when the re-stained sections were examined before reference to the drawing made from the phosphatase reaction, recognition of primordial germ-cells was difficult and a number escaped detection.

Embryos were fixed in cold 95 per cent. ethyl alcohol, dehydrated, semi-infiltrated in cellulose acetate, and infiltrated and embedded in 52°–54° C. M. P. Tissuemat. Complete serial sections were cut at 6 μ. Slides were exposed to the incubation mixture (Gomori, 1951) containing Fast Red Salt TRN at pH 9·4 for one-half hour. The mounting medium was glycerine jelly.

It is not feasible in practice to trace individual germ-cells, when they are numerous, serially through the sections. The error due to their diameter (6·25–12·5 μ) and the section thickness was therefore minimized by counting in alternate sections, except where so few cells were present that they could be followed serially. The lowest germ-cell totals, which are the critical cases for identifying defectives, therefore represent a high degree of quantitative accuracy. All sections were studied, and locations as well as numbers of cells recorded.

No special comparison was made between germ-cell numbers in fertile heterozygotes (*W^{i}w*) and pure-recessive normals (*ww*). In the first study, *ww* × *Ww* and *ww* × *W^{v}w* offspring were compared with *ww* × *ww* litters (Mintz & Russell, 1957). While 50 per cent. heterozygotes were expected in the former cases, the germ-cell totals were within the all-*ww* range, suggesting that there is no influence of those mutant alleles in a single dose.

The *ww* × *ww* controls collected earlier were on an isogenic C57BL/6 background. No matings of these genotypes in C3H mice were included in the present survey for comparison with crosses segregating defectives. Results nevertheless appear clearly to warrant the conclusion that two populations, normal and abnormal, segregate in *W^{i}w* × *W^{i}w* matings.

RESULTS

Germ-cell totals in embryos from *W^{i}w* × *W^{i}w* are given in Table 1. At *8 days*, the numbers form a continuous series in which adjacent members are no more than 13 cells apart and no discrete population of low numbers is distinguishable. The range (24–98) is comparable to that found in 8-day *ww* × *ww* embryos on a C57BL/6 background. All cells are found in their normal locations (Plate 1, fig. A).

At *9 days*, however, two counts (70, 71) at the low end were separated by a gap of 111 cells from the next highest member of the series, while the remainder showed a maximum difference of only 39 cells between successive frequencies (except for the highest, which was slightly more advanced developmentally).

The first two are therefore regarded as constituting a distinct population. The range (70–446) extended below that of the previously described *ww* controls (149–379).

TABLE 1

Total numbers of primordial germ-cells in embryos from matings of $W^jw \times W^jw$

Age of embryos	*Germ-cell totals**
8 days	24, 31, 40, 50, 55, 68, 69, 77, 85, 92, 98
9 days	*70*†, *71* (2), 182, 206, 206, 245, 446
$10\frac{1}{8}$ days	*70*, *110* (57), *155*, 583 (73), 1,051 (130), 1,117 (4), 1,306 (139), 1,852 (118), 2,217 (260)

* Numbers in parentheses represent cells, out of the preceding total for that embryo, found in positions outside of the normal migratory route described.

† Counts selected as indicating the presence of the defect, and therefore the genotype *WiWi*, are shown *in italics*.

At *$10\frac{1}{6}$ days*, three embryos were similarly found in a group having small numbers of germ-cells (70–155), indicating virtually no mitotic increase over the frequency at 8 days. (These cannot be compared precisely with the *ww* controls at 10 days because of the age discrepancy.) The individual with the lowest number of cells (583) which was still classified as normal was slightly retarded in development. The disparity between cell numbers in presumably defective and normal groups was even greater than at 9 days.

Locations of primordial germ-cells along the route from yolk sac to germinal ridges were normal for embryos with the larger cell totals at 9 and $10\frac{1}{6}$ days (Plate 1, fig. B; Plate 2, fig. D). In those with small totals, on the other hand, a disproportionate retardation in migratory progression was observed (Plate 1, fig. C; Plate 2, fig. E).

Totals of all cells found to be situated in places outside the normal migratory path are indicated in parentheses in Table 1. The number of such cells at 9 days is two, and they range from 4 to 260 at $10\frac{1}{6}$ days. They comprise between approximately 0·4 and 50 per cent. of the total germ-cells in a given individual, with the majority in the range of 6–12 per cent. Although the single highest incidence occurs in a putatively defective individual, there appears to be no consistent correlation between total number and fraction of that total which are ectopic. Three of the five defectives contain no ectopic cells at all. By far the most frequent location of 'lost' cells is in the outer skin ectoderm, usually in posterior regions of the body or in the tail. Their fate is unknown and they will be the subject of another study.

Combining the data for 9 and $10\frac{1}{6}$ days, where a group deviating from the normal is detectable, we obtain 5 cases out of 16 in the deviant class, which differs by only one case from the theoretical 4/16.

DISCUSSION

The presence of normal numbers of primordial germ-cells in all embryos at 8 days suggests that the W^j mutation, in homozygous condition, has no effect on initial germ-cell formation, although homozygotes might conceivably be lacking in this sample. Among 24 additional embryos from litters expected to segregate homozygotes (of W or W^v), a defective group similarly failed to be evident at 8 days (Mintz & Russell, 1957).

Deviation from the migratory path leading to the germinal ridges is also inadequate to account for subsequent sterility of the gonads.

The principal abnormality produced in the W^jW^j genotype is a marked failure of germ-cells to increase in number during the migratory period. This defect is first observed in the 9-day embryo, but the basis thereof may already exist at an earlier stage. Its manifestation is exaggerated with time as cell totals of normal and mutant individuals become more widely separated. In the study of homozygous mutants of W and W^v (Mintz & Russell, 1957), the same deficiency was observed, and a tabulation of degenerating germ-cell fragments (still stainable for alkaline phosphatase) suggested that low numbers of intact cells did not primarily represent a normal mitotic rate combined with excessive mortality. It is possible, however, that the few germ-cells found in homozygotes might eventually prove to have a limited viability. This mitotic insufficiency, from whatever cause, is accompanied by retarded migration in homozygotes of all three alleles.

In comparing effects of W^j with those reported for W and W^v, it is particularly striking that all of the mutant genotypes involved show similar ranges of germ-cell numbers at the ages studied. At 0–28 days post-natal, however, the gonads of surviving homozygotes may be ranked, revealing the most severe defect in WW and graded severities decreasing from WW^v to W^vW^v, corresponding to the intensity of anaemia in the same animals (Coulombre & Russell, 1954). (Quantitative observations on post-natal W^jW^j sex glands are not yet available.) The evidence suggests that the seriation of effects is established between the end of germ-cell migration and the time of birth, starting at about the period of sex differentiation (12 days).

The relative times of onset of anaemia and sterility are important for analysis of the pleiotropic action of the W-alleles. The first indication of abnormal haematopoiesis is seen in the $12\frac{1}{2}$-day embryonic livers of W and W^v homozygotes (Mirsky, 1949), while the earliest visible effect of W, W^v, or W^j on germ-cells occurs at 9 days. Therefore the sterility does not appear to be a secondary consequence of the anaemia in these animals. Borghese (1955) reached this same conclusion on the basis of continued sterility of 12-day WW gonads after explantation to a nutrient medium *in vitro*. It should be pointed out, however, that the discovery that the 12-day WW gonad is already severely deficient in germ-cells (Mintz & Russell, 1955) must modify the interpretation of such evidence;

retention of the sterile character is, instead, an indication of the irreversibility of the defect after its appearance. Russell, Murray, Small, & Silvers (1956) have transplanted 12–16-day WW and W^vW^v fetal gonads to normal adult spleen, and supported our embryological findings: genotypically autonomous development continued in a non-anaemic environment.

Even if an undetected defect in yolk sac haematopoiesis should exist at 8 days, and therefore earlier than the observed germ-cell defect, it is unlikely that it would have a physiological influence on the activities of the germ-cells because of the lack of a functional embryonic circulation at that time. It is possible that a fourth type of tissue could be influenced by the primary action of these genes and would then secondarily affect blood, pigment, and germ-cell development, but supporting evidence remains to be discovered. The possibility must also be entertained that a generalized metabolic error in all cells of the defectives provides an unfavourable environment for development of the three selected cell types. Until data are presented on this point for the cells and stages in question, we may incline toward the alternative that the pleiotropic effects stem from a single gene-mediated alteration to which certain kinds of cells are peculiarly vulnerable because of their own special activities (see discussions in Grüneberg, 1938, and Russell, 1949). It is not possible to speculate fruitfully at the present time on possible common requirements shared by these particular cells.

A number of investigators have regarded the so-called germinal epithelium of the gonad as the source of primordial germ-cells; some (including Everett, 1943, and Chiquoine, 1954, in work on the mouse) have found the germ-cells arising extra-regionally and migrating to the gonad. This much-debated embryological controversy was regarded as resolved by the first *experimental* verification of the extra-gonadal origin of germ-cells in a mammal; the 'experiment' was a genetic one, in which ratios of presumptively sterile embryos from heterozygous parents would be expected to conform to Mendelian expectations and known incidence of steriles at birth (Mintz & Russell, 1955, 1957). Validation depended upon frequency of certain alkaline-phosphatase-positive cells, known to be neither blood- nor pigment-cells, and corresponding in location with a path toward the germinal ridges. In a large sample, the frequency of individuals having small numbers of these cells conformed closely to the theoretical outcome. The conclusion was therefore reached that the cells described were actually the primordial germ-cells, and that their origin is extra-gonadal. This conclusion is fully supported by the additional evidence obtained in matings of $W^iw \times W^iw$.

SUMMARY

1. Twenty-seven mouse embryos at 8–10$\frac{1}{8}$ days from matings of $W^iw \times W^iw$ were examined in order to establish the embryological basis of the germ-cell deficiency found in the W^iW^i genotype at birth. Elective staining of primordial germ-cells was obtained with the azo dye coupling histochemical technique for

alkaline phosphatase. Numbers and locations of these cells were recorded in complete serial sections.

2. Formation of germ-cells occurs, as in normals, in all members of the 8-day group. At 9 days and later, during gonial migration from yolk sac toward germinal ridges, two distinct populations of individuals are recognizable. In one, the number of germ-cells increases; in the other, totals remain very low. The latter would be expected to characterize the W^jW^j class. Observed incidence of such cases was close to the theoretical frequency of 25 per cent. defective offspring. These findings support the earlier proof (Mintz & Russell, 1955, 1957) that the cells observed are the primordial germ-cells and are of extra-gonadal origin.

3. In defectives, migratory retardation of germ-cells accompanies their mitotic insufficiency.

4. The effects of W^j are compared with those of the alleles W and W^v, and the significance of the study for the problem of pleiotropic gene action is considered.

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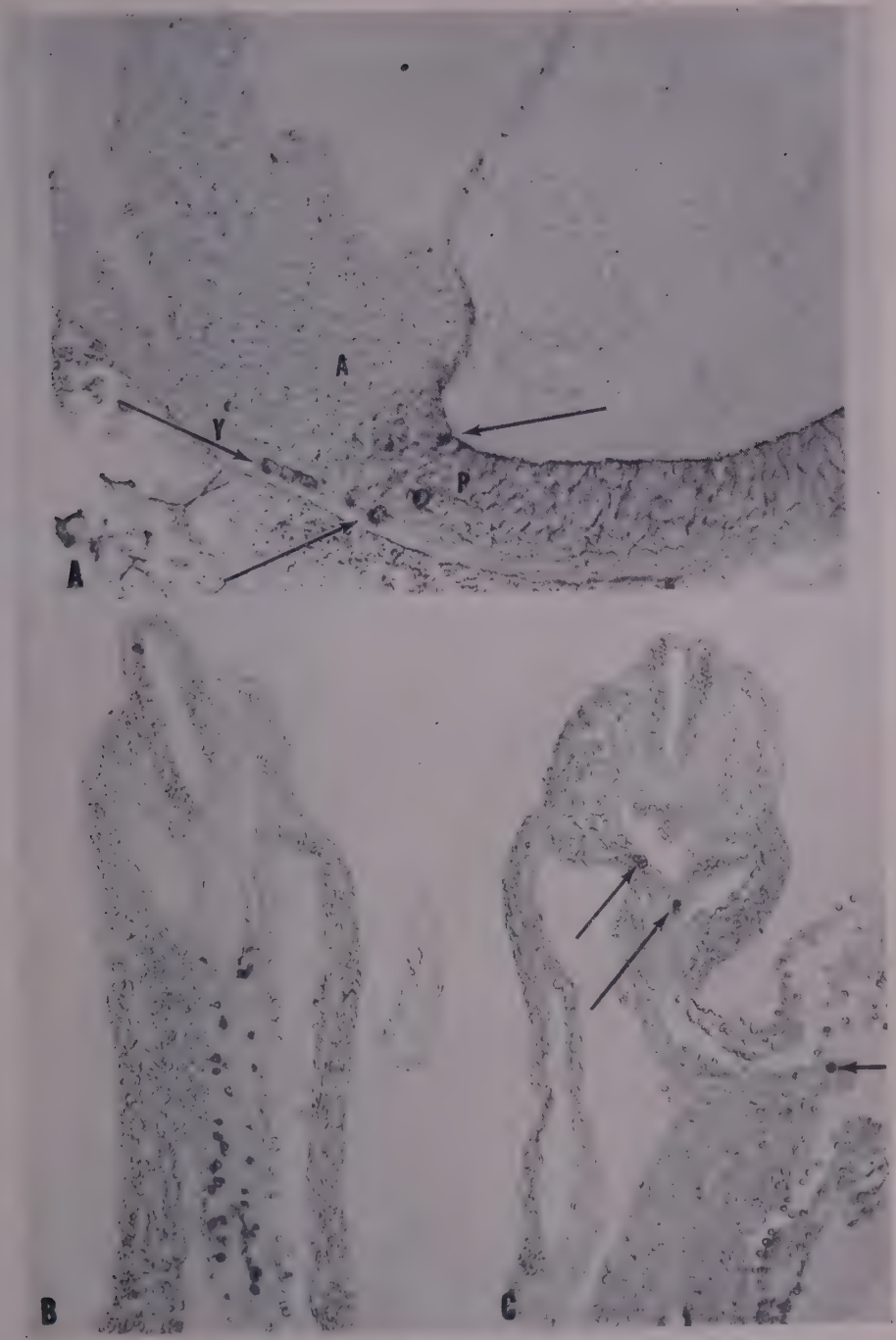
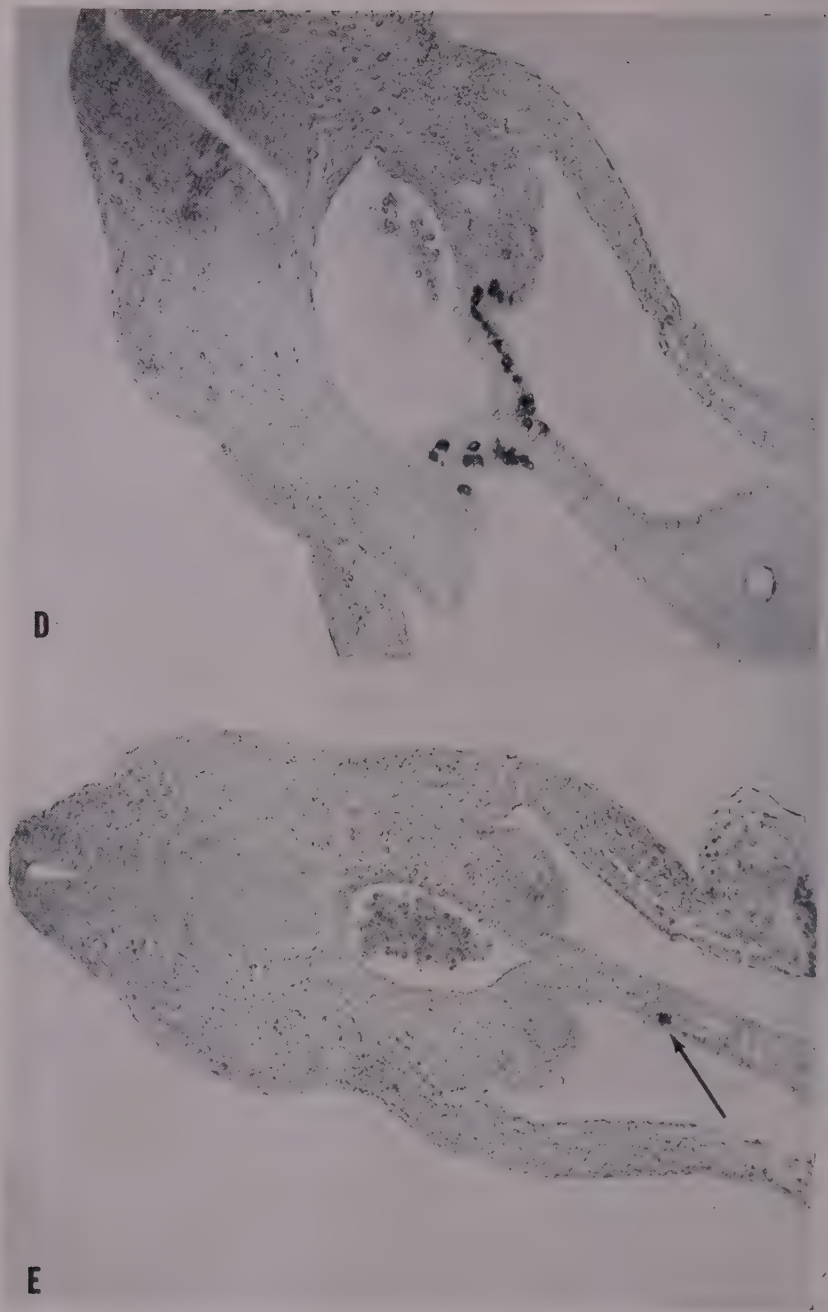


B. MINTZ

Plate 1



B. MINTZ

Plate 2

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Sagittal section through allantois (A), primitive streak (P), and yolk sac (Y), at 8 days. Primordial germ-cells (arrows) are located in the yolk sac and at the posterior end of the primitive streak. ×230.

FIG. B. Normal (*? w*) embryo at 9 days. Many primordial germ-cells in gut and dorsal mesentery, and near dorsal aorta. Cross-section. ×115.

FIG. C. Defective littermate (*WiWi*) of the 9-day embryo shown in Fig. B. Three germ-cells (arrows, left); one (arrow, right) retarded in migration, in yolk stalk. Cross-section. ×115.

PLATE 2

FIG. D. Normal (*? w*) embryo at $10\frac{1}{8}$ days. Many primordial germ-cells at coelomic angles and in medial parts of germinal ridges. Cross-section. ×115.

FIG. E. Defective littermate (*WiWi*) of the $10\frac{1}{8}$-day embryo shown in Fig. D. One germ-cell (arrow) in dorsal gut mesentery. Cross-section. ×115.

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The Effect on Embryogenesis of a Sex-linked Female-Sterility Factor in *Drosophila melanogaster*

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WITH TWO PLATES

INTRODUCTION

WADDINGTON (1956) has called attention to the importance of the relation between the structure of the egg and hereditary factors which determine characters in the developing organism: 'When we discuss the eggs of the different kinds of animals, we . . . find that the eventual origin from which the whole later development springs is the orderly arrangement of essential parts of the ovum. We must therefore enquire a little more deeply how this arrangement is brought about. In particular, what is the relation between it and the hereditary factors or genes which determine the detailed character of the adult organism?'

Although, in most animals, no such relations have been carefully studied (with the exception of direction of coiling in *Limnea*) there exists in *Drosophila* a class of female-sterility genes or factors in which females are sterile because the egg cytoplasm will not support the development of a viable zygote (Lynch, 1919; Merrell, 1947; Counce, 1956 *a*, *b*, *c*); e.g. when a female heterozygous for the gene is mated to a mutant male, females homozygous for the factor develop into adults; however, when these homozygous mutant females are mated, their offspring never develop to an adult stage. Apparently many such factors exist in *Drosophila*—an analysis of eight II chromosome female-sterility factors with no visible effects and chosen at random, produced three which were of the above type (Counce, unpublished). It should therefore be possible to collect considerable information as to the role of the egg cytoplasm and its relation to hereditary factors.

An advantage of such mutants is the existence of a viable homozygote for studies in developmental physiology, such as processes of ovary formation and egg development, and the developmental history of phenotypic differences when they exist. There is also a technical advantage over recessive lethal factors

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causing embryological death, for from female-steriles one can obtain 100 per cent. zygotes or eggs which are affected, rather than 25 per cent. This can be of great value in experimental studies of mutants, such as respiration rates, chromatographic differences, electrophoretic studies, &c., where it is sometimes necessary to utilize large numbers of eggs.

This paper deals with the effects of a sex-linked female-sterility factor, *nasrat*^{A} (*fs*^{nasA}) on the development of the embryo in eggs from homozygous mutant females mated to mutant males.

MATERIALS AND METHODS

The sex-linked sterility factor, *fs*^{nasA}, was found by Dr. Gamal Nasrat during the course of experiments on chemical mutagenesis carried out at the Institute of Animal Genetics, Edinburgh. There are no visible characters which distinguish it from the wild type, and it is associated with no gross chromosomal rearrangements (Dr. H. Slizynska, private communication). Linkage tests show that the factor is closely linked to *scute* (1·0±). All homozygous females are sterile; mutant males are fertile with non-mutant females. No analysis has been made of possible effects of the factor in heterozygotes. The stock is maintained against the Müller-5 chromosome.

The following description of embryogenesis is based on a study of serial sections of 647 embryos and eggs, from matings of *fs*^{nasA} homozygous females to *fs*^{nasA} males. At a given temperature, the rate of development in *Drosophila* embryos is quite constant, and it is possible to obtain embryos at various developmental stages by making timed collections of eggs and allowing them to develop to the desired stage. All developmental times and stages correspond to those given in Sonnenblick (1950) and Poulson (1950).

Detailed descriptions of methods of egg collection and histological techniques may be found elsewhere (Counce, 1956 *a*, *b*, *c*). Briefly, eggs are collected on agar lids over a 1-hour period from rapidly laying females 4–8 days of age. They develop at 25° C. until the desired stage is reached, and are then fixed. Routine methods of dehydration and embedding are used. Sections are routinely cut at 3–5 μ and are stained with Heidenhain's iron haematoxylin without counterstain.

RESULTS

Only offspring from crosses of mutant females to mutant males were studied.

Sectioned eggs could be divided into three categories: abnormal eggs which contained little or no yolk; early lethals—embryos which ceased development before the beginning of primary differentiation; and late lethals—embryos which developed beyond the first critical period and formed recognizable larval structures.

A. *Abnormal eggs*

Approximately half of the eggs produced by the females used in this study were yolkless or nearly so. All females laid normal and abnormal eggs. Such eggs are very white and extremely opaque when seen on the agar lids used for egg collection, and tend to collapse even when gently touched with a brush or dissecting needle; frequently their shape is abnormal. They are almost impossible to prick for fixation without severe damage. If they are left on the agar lids for more than 4 hours after deposition they are usually distinguishable from eggs which contain normal amounts of yolk. Recognizably yolkless eggs were not collected for fixation, but at early stages they could not always be distinguished from embryos in early developmental stages which are also opaque, and were therefore included among the sectioned material.

One hundred and nineteen yolkless or almost yolkless eggs were sectioned. Of these, 90, or about 75 per cent., were completely devoid of yolk, while the others had a few scattered yolk globules. In sectioned material the cytoplasm of such eggs is usually stringy and vacuolated, the size of the vacuoles varying from egg to egg. In some, small round objects are discernable which are not nuclei but which do not take the dark stain which Heidenhain's iron haematoxylin imparts to normal yolk globules, suggesting that abnormal yolk may be deposited.

Fertilization and subsequent nuclear divisions can occur in these abnormal eggs. Nuclei are found in approximately half of the sectioned eggs. Although there may be several thousand nuclei present in the egg, they never migrate to the surface to form a blastoderm, and they soon become pycnotic.

The inner membrane (or plasma membrane) of fs^{nasA} eggs may be abnormal or weak in the region of the micropyle, for frequently the ooplasm may 'leak' out at the anterior end and flow into the micropyle. In some, so much material flows out that it fills part of the space between the vitelline membrane and the chorion.

It is probably this same weakness which accounts for the difficulties encountered when we attempted to make films of the development of fs^{nasA} embryos. All our attempts failed because the eggs always collapsed within an hour or two of being placed either in water (in which normal and other mutant embryos develop without difficulty) or in insect saline (0·75 per cent.). Leaks were also found in sectioned eggs in which development was proceeding.

Out of the total number of eggs examined, 48, or 7·4 per cent., were classified as unfertilized. This figure is probably exaggerated, for some early lethals are no doubt also included in the group. It is not always possible to determine after 6 hours whether the gamete nuclei have failed to unite or whether development has ceased shortly after this has occurred. The difficulty is compounded because certain cytological abnormalities typical of these eggs (see below) make it even more difficult to ascertain if there are nuclear structures present.

B. *Early lethals*

Of the 480 eggs in which there was recognizable development, 94, or about 20 per cent., ceased to develop before the beginning of primary tissue differentiation, the majority (90/94) ceasing to develop during the first 2 hours (nuclear cleavage stages). These were classified as 'early lethals'. Their distribution was as follows:

Abnormal maturation divisions . .	7
Abnormal syngamy or first cleavage .	12
Abnormal early cleavage (2nd–4th) .	25
Abnormal mid-cleavage (5th–7th) . .	11
Late cleavage—blastema . . .	35
Blastoderm—gastrula	4

It is probable that this is an underestimate of the number of early lethals because some of the cleavage and blastoderm stage embryos might actually have ceased development in a short time had they not been fixed, and those embryos which die during maturation, syngamy, or early cleavage and were not fixed until late in development will probably be classed as unfertilized eggs.

Early lethals are characterized by a vast array of cytological abnormalities and of abnormalities in nuclear distribution. Because the cytological abnormalities found in fs^{nasA} embryos are of special interest, they will be discussed in a separate section.

Micropylar leaks were found in nine of the early lethals.

C. *Late lethals*

Cleavage (0–1¾ hours). Those fs^{nasA} embryos which successfully pass the first 2 hours of development usually continue to develop until the end of embryonic life. However, they become abnormal early in development, and as development continues, these abnormalities become magnified.

Although the existence of abnormalities in early cleavage stages indicate that development may become abnormal very early in embryogenesis, it can remain normal through at least the 4th cleavage division.

By the 6th–8th divisions, however, development has become abnormal in almost all embryos. There were available only a limited number of embryos at this stage and all were abnormal, but the occurrence of some normal blastoderm stages indicate that development is not invariably abnormal by this stage. Abnormal embryos are characterized by abnormalities in migration of nuclei, lack of mitotic synchrony, and aberrant cytological features (see below).

Blastula stages (1¾–3 hours). In the normal embryo, nuclei migrate to the surface at the 9th cleavage division and divide there in synchrony three more times. The cell-membranes then form from the outer surface inward creating a cellular blastoderm. During this time the yolk granules are becoming

concentrated in the centre. The pole cells are differentiated at the posterior end, usually during the 9th and 10th divisions.

One hundred and seventeen *fs*^{nasA} embryos at this stage of development were studied. These included embryos from the time of the 1st divisions at the periphery (blastema) to the formation of the cell-membranes (cellular blastoderm). Classification is not always simple in early blastema stages, for abnormalities in nuclear distribution and mitotic synchrony result in embryos which are at the 1st or 2nd blastema divisions at the anterior end, but which have the typical appearance of late cleavage stage embryos at the posterior end. About 20 per cent. of the embryos at this stage are apparently developing normally.

TABLE 1

Abnormalities in blastula and blastoderm stages of late lethal embryos

n	*Per cent.*	*Nuclei out of phase*	*Cytological abnormalities*	*Abnormal distribution of nuclei or incomplete blastoderm*
34	41	+	+	+
20	25	−	+	+
8	10	−	−	+
6	7	+	−	+
6	7	−	+	−
5	6	+	+	−
4	4	+	−	−

\+ abnormality present in embryo.
− abnormality not present in embryo.

Nuclear divisions during cleavage and the syncytial blastoderm stage are synchronous in normal embryos, with the exception of those of the yolk nuclei and the pole cells. In *fs*^{nasA} embryos over half of the embryos in these stages show departures from this synchrony. Moreover, there is a general pattern of differences in synchrony which occurs in 90 per cent. of the affected blastula stages. The yolk nuclei tend first of all to depart from synchrony earlier than is normal, and frequently show many cytological disturbances. The surface of the embryo shows three (sometimes four) different areas which are clearly delimited from one another (Plate 1, fig. A). The posterior $\frac{1}{4}$–$\frac{1}{3}$ is usually most severely affected, the area being always somewhat broader ventrally, and in 40 per cent. of the embryos which show abnormal synchrony, the posterior region is the only visibly affected surface portion. This region is frequently in late stages of cleavage while the rest of the embryo is in the 1st or 2nd stage of blastoderm cleavage. Later this posterior region may not form a complete blastoderm. This is due to two different processes: in some embryos nuclei do not migrate to all areas, while in others there is an indication of grave nuclear disturbance and of nuclear breakdown after they reach the surface. The anterior tip, when it is affected, is usually one cleavage stage behind the adjacent sections, although one also finds embryos

in which nuclear migration is not completed. Nuclear breakdown may also occur. The two central regions may or may not show differences from each other. Differences, when they do exist, are usually in nuclear size; in some instances they are in different phases of cleavage, the anterior of the two apparently the more advanced.

The boundaries between the different regions are very sharp; usually the first one or two bordering cells are markedly abnormal. This is important since normal embryos which are fixed in stages when synchronous mitoses are occurring show a fixation gradient, and one must be able to distinguish between these gradients and actual differences. The borders of these regions also roughly coincide with the positions of the abnormal furrows which form during gastrulation.

Many of the blastoderm embryos showed cytological abnormalities. Abnormalities in mitotic phase, division figures, and abnormal distribution of nuclei may be related and are found in different combinations. Table 1 shows the relations between these three abnormalities based on a detailed analysis of eighty-three embryos.

Because of irregularities in division and distribution of nuclei, cell-membrane formation is usually abnormal in fs^{nasA}. The blastoderm wall varies in thickness, usually being narrower at both ends than in the central regions. When the blastoderm wall is incomplete at either end there is usually a broad band of cytoplasm containing a mixture of yolk globules and nuclei which runs to the tip (Plate 1, fig. B). The size of nuclei making up the cellular blastoderm also varies. In some regions the nuclei are much smaller and more closely packed than in normal embryos, suggesting that in some regions an abnormally large number of nuclei may form prior to cell-membrane formation.

In normal embryos the posterior polar plasm is visibly differentiated from the rest of the periplasm by the presence of discrete granules which were called 'the germ-cell determinants' by Hegner. Their exact function is not clear; however, most of them are included in the cytoplasm of the pole cells during their formation. These discrete bodies are only rarely observed in fs^{nasA} embryos.

In approximately half of the abnormal blastula embryos, the pole cells do not form; they form in the remainder but there is always some breakdown of pole cells rather soon after they form.

After the formation of cell-walls at about 3 hours in the normal embryo, some of the pole cells migrate into the interior of the embryo; those which remain at the posterior end are later carried passively into the embryo by the developing posterior mid-gut rudiment. In fs^{nasA} embryos, pole cells migrate into the interior at 3 hours; apparently all of the pole cells which are not too abnormal may enter at this time (Plate 1, fig. C). The second movement of pole cells never occurs in fs^{nasA} because of abnormalities in the formation of the posterior mid-gut rudiment.

Distribution of yolk is rather abnormal, the globules being concentrated peripherally while large vacuoles in the centre contain only tiny droplets con-

nected by cytoplasmic threads (cf. Counce, 1956*a*). Frequently large clumps of highly abnormal yolk nuclei are found within these vacuoles.

Eighteen of the embryos had material leaking into the micropyle. In one embryo, it was apparently the only abnormality.

Gastrulation and germ-band extension ($3\frac{1}{2}$–$4\frac{1}{2}$ hours). Gastrulation in the normal embryo consists of the formation of a long furrow on the ventral surface (the germ-band), followed shortly by the formation of an anterior cleft, the cephalic furrow. The posterior mid-gut rudiment then moves from the posterior region of the embryo around to the dorsal surface as the germ-band extends around to the dorsal side, the posterior mid-gut rudiment carrying the pole cells in its deepening cavity. The anterior mid-gut invaginates from the anterior ventral surface at about the same time. During this period the rudimentary embryonic membranes are also formed.

At this stage of development all fs^{nasA} embryos are characterized by the development of a deep cephalic furrow in the anterior region; a deep furrow, which forms from the ventral surface about one-fifth of the way from the posterior end, and, anterior to it, two slightly less pronounced lateral furrows running in a dorso-ventral direction. There are also several lateral subsidiary furrows, usually running from the dorsal to the ventral surface, and there may be a furrow anterior to the cephalic furrow.

Germ-band formation is delayed (or formation of the cephalic furrow is precocious); its formation may also be abnormal, and its shape is always severely affected by the presence of the many deep furrows in the embryo (Plate 1, fig. D). In some instances the connexion between the various regions of the embryo created by the furrows is so narrow that the germ-band may be only one cell layer in thickness. The furrows can apparently prevent the formation of the germ-band in some areas. In rare instances the germ-band may never form.

The posterior mid-gut invagination never moves from the ventral surface. Part of the major posterior furrow is probably the posterior mid-gut rudiment, the two lateral folds just anterior to it representing the lateral arms of the posterior mid-gut which are apparent on the dorsal surface of the living embryo as the rudiment moves forward. Its position on the ventral surface is anterior to that of the normal rudiment which also forms first ventrally and then moves over the posterior pole. In fs^{nasA} embryos this may be due to the failure of certain gross movements of the egg during cleavage and blastoderm formation (Imaizumi, 1954). This interpretation is given added support by the fact that the stomodaeum in fs^{nasA} embryos forms further anterior than is normal.

No evidence of proctodaeal formation has been found at any stage in the development of fs^{nasA} embryos.

Cell disintegration has already begun by this time, usually at the posterior end among those nuclei involved in large masses of cytoplasm when blastoderm formation is not completed, and also in the anterior end where cells have remained in the non-cellular cytoplasm.

Pole cells were identified in only 8 out of 64 embryos, undoubtedly because it is difficult to identify them positively at this stage when they may be isolated single cells. None are ever included in the posterior mid-gut invagination.

The embryonic membranes, or elongate cells which closely resemble them, form in the abnormal embryos at this time, covering the entire region from the cephalic furrow back to the posterior lateral folds, a region more extensive, however, than that usually covered by these membranes.

In fs^{nasA} embryos the posterior tip is frequently attenuated, and this abnormal shape first becomes obvious during the late stages of gastrulation.

Leaks were observed in 10 out of 64 embryos.

Histogenesis and primary organogenesis (5–12 hours). Between 5 and 12 hours in the normal embryo the basic tissues become differentiated and the primary organs formed. At the end of the period three major morphogenetic movements occur—shortening of the germ-band, dorsal closure, and head involution. These developmental stages in fs^{nasA} embryos carry the strong imprint of earlier abnormalities.

The formation of the stomodaeum is always affected; it forms at the anterior tip instead of on the ventral surface and is usually very short. No stomodaeum was formed in 13 out of the 96 embryos studied at these stages.

Formation of the anterior mid-gut may also be affected, the rudiment frequently consisting of fewer cells than normal, and in six embryos it failed to form at all. Its position in regard to the stomodaeum is frequently aberrant.

The posterior mid-gut could be definitely identified in only twenty embryos. However, it is not easy to identify during this period for its structure may be modified considerably, and the cells at this time bear a strong resemblance to some of the mesodermal elements.

The results of earlier abnormalities in the formation of the germ-band are now clearly apparent. It was lacking in 6 embryos, incomplete in 26, and showed little or no differentiation into its component parts in 21. Degeneration of the mesoderm may begin by this time; pycnotic or disorganized mesodermal cells appeared in thirteen embryos. In less severely affected embryos the differentiation of the mesoderm into somatic and visceral musculature and fat-bodies may be detected.

Although the differentiation of the hypodermis is very good, its development reflects abnormal blastoderm formation. It was incomplete in $\frac{2}{3}$ of the embryos at these stages: of these, 57·6 per cent. were incomplete on the posterior ventral surface, 16·7 per cent. at both anterior and posterior ends, 19·7 per cent. at the anterior end only, and the remaining 6 per cent. in other regions. Tracheal pits form (perhaps earlier than in normals) sometimes on the inner edges of the deep clefts which continue to divide the embryo. It is apparently impossible for the tracheal rudiments to elongate to any considerable extent, or to join to form the long main trunks. The salivary glands, which are also ectodermal in origin, may be identified in older embryos in this group, but their distortion and

displacement resulting from the abnormal furrows makes it difficult to identify them accurately before they enter their secretory phase at 12 hours. The rudiments apparently originate very near the boundaries of the cephalic furrow.

In some embryos segmentation is superimposed over the major furrows, but segments may appear slightly later than normal.

Pole cells were identified in 38 of the 96 embryos. As the embryos increase in age there is aggregation of the pole cells during gonad formation and they are more easily identified: e.g. at 5 hours they were identified in only 2 out of 10 embryos, as compared with 7 out of 14 at 10 hours.

The major morphogenetic movements which take place during this period are all affected in the abnormal embryos. It is doubtful whether any shortening of the very abnormal germ-band occurs. Dorsal closure of sorts takes place, probably by the transformation of the embryonic membranes or the embryonic membrane-like cells which form over the long central portion. The head segment —even the head ectoderm—may fail to develop, and involution is usually abortive or does not occur. Only a few embryos have been observed in which considerable involution has taken place, and there is never the extension of the segments at the end of involution which there is in normal embryos (Ede & Counce, 1956).

Almost 9 out of 10 embryos between the ages of 5 and 12 hours show signs of advancing deterioration, although rarely embryos up to the age of 10 hours may have no disintegrating cells. Disintegrating cells are most frequently found in the posterior region or the anterior region where nuclei are involved in masses of cytoplasm. It has been mentioned that breakdown of the mesoderm may be apparent by this time.

Only two embryos of this stage were observed with leaks at the anterior end.

Final pattern of damage. Following the major morphogenetic movements in the normal embryo, the period from 12 to 18 hours is largely devoted to cellular differentiation in the various organs and tissues until they reach the stage of differentiation characteristic of early first instar larvae. Hatching occurs at about 20 hours.

Although development first becomes abnormal in fs^{nasA} embryos at a very early stage, and cells begin to degenerate almost immediately after their formation, monster embryos with many recognizable larval characteristics may be formed at the end of embryonic development. None of these ever emerge completely from the embryonic envelopes, although some may succeed in rupturing the membranes and partially emerging.

These monster embryos may be divided roughly into two groups—those in which segmentation has been at least partially superimposed over the major furrows ('segmented' embryos) (Plate 1, fig. E), and those in which only the furrows are still evident, resulting in an embryo consisting of four or five sections ('compartment' embryos) (Plate 1, fig. F). These two groups gradually grade into each other, but as a rule those with only the major furrows are more

abnormal in all respects: for example, only 3/24 (12·5 per cent.) of the 'compartment' embryos showed differentiation of mesodermal tissues, and mesodermal tissues were completely lacking in 4/24 (16·7 per cent.), while the comparative figures for 'segmented' embryos were 35/58 (60·3 per cent.) and 0/58 (0·0 per cent.).

Formation of the gut is always affected. No embryo has ever been observed which had either a hind-gut or Malpighian tubules. Owing to the abnormal origin of the posterior mid-gut invagination, the structure of the mid-gut is always abnormal. However, in 18/56 of the 'segmented' embryos, the two rudiments of the mid-gut formed a more or less complete structure around the main yolk mass; this never occurred in any of the 'compartment' embryos, where the middle regions of the embryo usually consist of a mass of yolk and undifferentiated or disorganized and pycnotic cells covered by hypoderm-like cells. Rudiments of the posterior mid-gut were present in 41·6 per cent. of the 'compartment' embryos and in 76·8 per cent. of the 'segmented' embryos. In many embryos the anterior mid-gut developed certain characteristic structures, such as the gastric portion of the proventriculus and the gastric caecae. Union with the abnormal stomodaeum or oesophagus did not always occur, but a recognizable proventricular structure sometimes formed. The anterior mid-gut was completely lacking in 12·5 per cent. of the 'compartment' embryos but in only 2 per cent. of the 'segmented' embryos.

The mesoderm is severely affected. It is capable of differentiation into its major components—fat-bodies, somatic and visceral musculature—in some embryos. The spatial displacements which occur in all embryos make it impossible for complete normal differentiation of either the somatic or visceral musculature to occur, and usually these elements consist of large clumps of partially differentiated cells with slightly different staining reactions and morphology (Plate 2, fig. G). The somatic elements of the gonad can develop.

In two embryos in which head involution was almost normal the ring gland and aorta were formed as well as the frontal sac and Guirlanden cells, indicating that the potential for differentiation into these structures exists, but the circumstances which permit it to be realized occur only rarely in fs^{nasA} embryos.

Development of the nervous system is also affected by the abnormal furrows, and it usually consists of isolated clumps of cells, differentiation usually being best in the brain region. In 'compartment' embryos, recognizable ganglion cells are never found in the central yolk-filled compartment, but probably contribute, along with mesoderm cells, to the mass of undifferentiated and frequently pycnotic cells found there.

Hypoderm formation is almost never complete (mirroring earlier developmental events) although its differentiation is usually good. It is most frequently incomplete posteriorly and ventrally where nervous tissue and clumps of somatic mesoderm are exposed to the surface. In the head region the brain is often exposed, a common event in *Drosophila* mutants when head involution is not

normal (see discussion in Counce, 1956*b*). The main tracheal stems are never formed but isolated bits of trachea with larval differentiation are found in almost all embryos, and posterior spiracles differentiate in a good number. At least one salivary gland formed in most embryos, although the position of the gland or glands was usually distorted. Many contained the normal secretion which stains with Heidenhain's, indicating that their functional capacity is probably not impaired. Rarely cells which had not invaginated to form the sac-like gland also showed secretory products.

Pole cells or gonads were identified in over half of the 'segmented' embryos and in 37·5 per cent. of the 'compartment' embryos; this is approximately equal to the number of embryos in which pole cells are formed. In a few instances three small gonads were formed, while in others a single pole cell apparently organized a 'gonad' around itself (Plate 2, fig. G). Frequently, only a single gonad formed, but isolated pole cells might be apparent in other regions of the same embryo. Usually the gonad or gonads were in close contact with cells of the posterior mid-gut rudiment.

Cell disintegration is found at this stage in all 'compartment' embryos and in over 80 per cent. of the 'segmented' embryos. In some 'compartment' embryos either the posterior or anterior end may be devoid of cells; these areas resemble the appearance of unfertilized and disintegrating eggs. These are undoubtedly embryos in which large areas of the blastoderm were left uncompleted by the failure of cells to migrate or by the disintegration of cells before a blastoderm formed.

No leaks were observed in these embryos, but at this stage their presence could be masked by the increased breakdown of cellular material.

Cytological abnormalities. Cytological abnormalities occur with a high frequency in fs^{nasA} embryos (cf. Table 1). They first appear during cleavage stages. Nuclei of various sizes form; polyploid mitotic figures are common, and polyploid and multinucleate cells occur in later stages. Nuclei may also clump together in single cytoplasmic islands. This and the common occurrence of polyploidy, spindle interference, and multipolar spindles may indicate that nuclei do not separate far enough at telophase. This is also suggested by the frequency with which nuclei in tandem are observed.

Many embryos appear to cease development in an active mitotic stage. In these embryos spindles soon take on a cloudy appearance, and although they retain their shape, the entire structure stains uniformly, no fibres being discernable. Large multipolar spindles are common; sometimes only clumps of chromatin can be detected distributed randomly within the spindle. In late stages (early death—late fixation) one may find only clumps of chromatin in the cytoplasm of eggs which have the same general appearance as unfertilized eggs. One embryo was found in which at least twelve spindles could easily be identified; however, there were *no* staining chromosomes with the exception of those in the metaphase plate formed by the polar body chromosomes (Plate 2, fig. H). The

number of cytoplasmic islands in the embryo would indicate that more mitotic figures should have been present. It is highly probable that this is not an isolated event. In some of the early lethals it was only possible to make out faint grey areas where spindles had formed; moreover, some embryos which were classified as unfertilized had well-developed discrete cytoplasmic islands (which 'normal' unfertilized eggs do not) although no mitotic figures or nuclei could be identified.

Centriole division may also be impaired, for in one embryo a single centriole was apparently functioning as a common pole for three spindles, while in another a nucleus with only a single centriole organizing spindle-fibres was found (Plate 2, fig. I).

Pole-cell nuclei frequently become pycnotic within a few minutes of their formation. This may apply to all the pole cells of an embryo or to isolated cells. The lack of granules in the pole cells has been discussed previously.

Pycnotic nuclei (in addition to those of the pole cells) are found in mesodermal tissues, and in nuclei in abnormal regions at the anterior and posterior ends. Pycnotic nuclei are also found in embryos when development ceases at late cleavage or during blastoderm formation. These embryos also have many other abnormal cell types.

The nuclear and mitotic abnormalities described above have frequently been observed in abnormal *Drosophila* embryos (see discussion in Counce, 1956*a*). In addition there is apparently another type of nuclear breakdown which has not been described in earlier publications. In the early stages of the development of these abnormal nuclei, prophase nuclei have large clumps of chromatin in the centre of the nucleus, some of it staining faintly (Plate 2, fig. J); unlike normal nuclei the peripheral areas are apparently devoid of chromatin. There is also a rather indefinable 'raggedness' about the chromatin or condensed chromosomes. Later, the chromatin appears to divide into two clumps, one which contains most of the darkly staining material while the other is made up of more faintly staining material (Plate 2, fig. J). These nuclei may undergo division but probably soon break down as figures of abnormal metaphase and anaphase would indicate (Plate 2, fig. K). (Figs. J and K are taken from the same embryo which is $1\frac{3}{4}$ hours after fertilization judged both from collection time and stage of development). At a later stage the nuclei take on a cloudy appearance, the nuclear sap apparently giving a positive reaction with Heidenhain's haematoxylin (Plate 2, fig. L). Such nuclei may be the forerunners of the faintly staining nuclei which are characteristic of the posterior end (Plate 2, fig. M), especially in the region directly above and to the sides of the pole cells. They are often so faint that only very careful study reveals their presence. Single cloudy nuclei may also be found in other regions of the embryo, especially during blastoderm stages. No cloudy nuclei have been observed later than early gastrula stages.

Yolk nuclei also behave very abnormally; they soon become very large, have an unusual staining reaction, and tend to clump together in long strips in the central regions of the embryo (Plate 2, fig. M).

DISCUSSION

Although the mutant factor *fs*^{nasA} has no visible effects in the adult, it has a very marked effect on the formation of the egg, and on the embryos developing in eggs from mutant mothers.

Over half the eggs from *fs*^{nasA} females contain abnormal amounts of yolk, and the structure of the cytoplasm is also altered. Abnormal eggs have been observed in other female-sterile mutants (Counce, 1956*c*), although there is a relationship between the type of abnormality and the genotype of the mother. The formation of such eggs is no doubt related to physiological disturbances in the mutant females. There is a possibility that the ring gland is in some manner involved, since it is known that a hormone produced by the gland is involved in the process of yolk deposition.

In the developing embryo the lethal effect of *fs*^{nasA} may be expressed either before blastoderm formation (early lethals) or, if this early epigenetic crisis is survived, development continues until a monster larva is produced which is not able to survive a second epigenetic crisis, that of hatching. Lethals with more than one critical period are not uncommon (Hadorn, 1955; Counce, 1956*a*). In the present instance all lethal individuals have a basic similarity in that all are characterized by abnormalities in division rate, in mitotic division and in cell structure. It is probable then that the two classes are closely related, although it is not clear why some survive the first critical period while others do not. The early lethals may develop in a cytoplasmic environment which is more abnormal —it may be that a threshold phenomenon of some sort is involved. Differences in development may be the result of variance between eggs from the same ovary, or perhaps of differences in age, environmental conditions in bottles, or the presence of modifying genes (see also discussion in Ede, 1957).

Whether early or late, the main effect of the gene is probably on the control of cleavage synchrony. Moreover, there appears to be a regular pattern in which disturbances of synchrony occur, suggesting the existence of stratification within the egg cytoplasm. It is not possible to determine whether loss of synchrony is the result of delay in some regions or an acceleration in others or a combination of the two. It is probable that it is the last mentioned which most frequently happens; it is also likely that the differences become magnified in successive divisions, for they stand out more sharply in the late blastema and cellular blastoderm than in the early blastema. At any rate, development of the embryo is severely altered in the 4th dimension, the odd pattern of delay and precocity occurring again in gastrulation and even during the period of histogenesis.

The rapidity and synchrony of cleavage divisions in *Drosophila* has been frequently discussed (e.g. Sonnenblick, 1950; Goldschmidt, 1955). Painter (1940) proposed that rapid division is possible because the nurse-cells furnish partially assembled 'chromosomal material' in the cytoplasm of the egg. During cleavage these partially assembled materials would be utilized rather than a complete

synthesis occurring. It is not difficult to invent explanations, with this theory as a basis, as to how this process of reassembly might go awry in female-sterile eggs and seriously affect cleavage. The nurse-cell nuclei are of course homozygous for the mutant factor and may provide in the egg cytoplasm abnormal amounts or kinds of 'building blocks'; alternatively, some substance which is necessary for the reassembly to occur may be affected.

The cytological evidence shows that the chromosomal cycle is severely affected. 'Chromosome-less' spindles found in one embryo, and the abnormal staining reactions of resting stage and prophase nuclei, as well as the 'raggedness' of some mitotic figures, may indicate that some stage in the cycle of chromosome duplication itself is basically affected. Other cytological evidence such as multipolar spindles, polyploidy, &c., although feasibly related to abnormalities in chromosome duplication, could also be related to abnormalities in function or structure of the spindle or centrioles. A third possibility is that the cytoplasm is in some way abnormal and prevents a potentially normal division from taking place (cf. Counce, 1956*a*).

It is important that we have in this mutant some possibility of carrying out further investigations on a cytochemical and physiological level which may give us some insight as to how these eggs differ from normal eggs. This in turn may help us to understand some aspects of the cleavage cycle in *Drosophila* and perhaps some of the processes involved in mitosis in general.

It is of interest that in addition to these abnormalities in synchrony and their fairly definite pattern in the egg, the polar granules which differentiate the posterior polar plasm from the rest of the periplasm are usually absent. What—if any—connexion there exists between them it is not possible to say, since the function and significance of the polar granules are not understood. One of us (S. J. C.) has studied a II chromosome female-sterility factor in which cleavage synchrony in the zygote becomes abnormal at a much earlier stage (3rd to 4th divisions) and in these eggs the polar granules are apparently normal.

The sex-linked lethal factor *X*2 (Ede, 1956) located near *forked* (56·7) has many striking similarities to the pattern of damage in fs^{nasA} embryos. In *X*2 the departure from normal development is usually first apparent in the gastrula stage, but in two blastoderm embryos Ede observed a striking abnormality: in both a complete blastoderm was formed, but the nuclei were of various sizes and in different stages of mitosis. As in fs^{nasA} embryos, the areas were sharply delimited (Plate 1, fig. A and Ede, Fig. 1). Gastrulation abnormalities are similar in that many deep abnormal furrows form, but in *X*2 they form only after extension of the germ-band begins. Invagination of the posterior mid-gut is affected in both but, again, the later effect of *X*2 is apparent, for the rudiment moves as far as the posterior pole before it invaginates. Subsequent developmental abnormalities are similar in the two, no doubt the consequence of early similarities. It should be pointed out that although there are marked similarities, the effects of the two factors vary both in *time* and *space*. *X*2 embryos usually show their first

abnormalities in early gastrulation, and the anterior end of the embryo is more severely affected than the posterior end—the converse of the effects of fs^{nasA} in which the posterior end is most severely affected (suggesting the possible existence of a posterior to anterior gradient in differentiation ?). The basic similarities of the two mutants in embryogenesis are probably based on abnormalities in mitotic rates which produce more surface cells than is normal.

The formation of gonads in fs^{nasA} embryos is of interest because of the questions concerning the mode of origin of the gonads in normal embryos. Earlier (reviewed by Sonnenblick, 1950 and Poulson, 1950) it was assumed that pole cells from the first period of migration were converted into yolk nuclei and those which were carried into the embryo in the posterior mid-gut later migrated out of the gut into the body-cavity and there formed the gonads. Poulson (1947, 1950) now believes that the latter cells contribute only to the formation of the middle mid-gut, and that the future germ-cells are pole cells from the first period of migration. These lethal fs^{nasA} embryos, admittedly very abnormal in development, support the interpretation of Poulson. In fs^{nasA} embryos, re-migration of pole cells (frequently all that form) at the first period has been observed in sectioned material; no pole cells are carried into the embryo during the second period because of abnormalities in posterior mid-gut formation. Yet gonads form in some fs^{nasA} embryos, showing that pole cells which enter during the first phase are capable of developing into gonads.

Waddington (1951, 1956) and Dalcq (1951) have discussed at some length the importance, for evolutionary processes, of mutations which alter the structure of the egg. Waddington has also pointed out that female-sterility genes in *Drosophila* provide favourable material for the investigation of such processes. Beatty (1949) has shown that such genes may alter the structure of the egg at any stage, and embryological studies of those affecting development in the egg (Counce, 1956 *a*, *b*, *c*) show further that development within the egg may be affected as early as the processes of fertilization or as late as the stage of larval differentiation of tissues.

Waddington (1951) voiced regret that in *Drosophila* no mutations '. . . had produced a change [in egg determination] which would allow a viable but abnormal development'. However, it was later shown (Counce, 1956*b*) that exactly such an effect was connected with the female-sterility gene *fused*. This gene is lethal to the zygote only if the somatic cells of the mother or the zygote nucleus do not contain the normal counterpart of the locus (Lynch, 1919; Counce, 1956*a*). It is possible, therefore, to obtain heterozygous females from (1) homozygous *fused* females mated to non-*fused* males, and (2) from non-*fused* females mated to *fused* males. In such heterozygotes the nuclear constitutions will be identical, the egg cytoplasms genetically different. Examination of adult female heterozygotes of these two kinds showed that abnormalities in abdominal segmentation were 30–40 times more frequent in females which developed from *fused* ooplasm; further, these abnormalities were connected with abnormalities

in musculature and segmentation patterns in the embryo. Undoubtedly, more examples will be found if someone looks for them, and it would seem that genes affecting the ooplasm and the establishment of predetermined patterns in the egg will be the most fruitful area for such a search. So many female-sterile mutants are known in *Drosophila* that the material for study is almost limitless, and it should be possible to provide a vast array of effects in time and space. Such mutations also have technical advantages for study by such techniques as paper chromatography (Hadorn & Mitchell, 1951), microrespiration studies (Chen, 1951), and cytochemistry of development (Yao, 1949, 1950) which have recently been used so successfully in studies of *Drosophila* development.

SUMMARY

1. Eggs from females homozygous for a sex-linked mutant factor, fs^{nasA}, will not support the development of a viable embryo.

2. Approximately half of the eggs produced by fs^{nasA} females are abnormal, containing little or no yolk.

3. Twenty per cent. of the embryos die during the first 2 hours of development. The remaining embryos develop into monster larvae which are not capable of hatching from the eggs.

4. The major effect of the factor is on the synchrony of cleavage and blastoderm mitoses. Moreover, the regular pattern in which disturbances occur suggests stratification of the egg cytoplasm.

5. Gastrulation and all subsequent developmental processes are abnormal. Germ-band formation and the development of mesodermal elements are strongly affected. The posterior mid-gut invaginates from the ventral surface of the embryo, and the proctodaeum and Malpighian tubules never form.

6. The possible effects of the factor on the processes of chromosome duplication during cleavage are discussed in connexion with Painter's theory.

7. The developmental effects of fs^{nasA} are compared with those of a sex-linked recessive lethal factor *X*2. The basic similarities of the two are probably the result of abnormal rates of mitotic division during blastoderm stages. Differences between the two suggest there may be a posterior to anterior gradient in regional differentiation of the embryo.

8. Gonad formation in fs^{nasA} embryos from pole cells which migrate through the blastoderm wall during the early stages of gastrulation supports Poulson's theory of gonad formation.

9. The broader implications of studies on female-sterility genes in *Drosophila* are briefly discussed.

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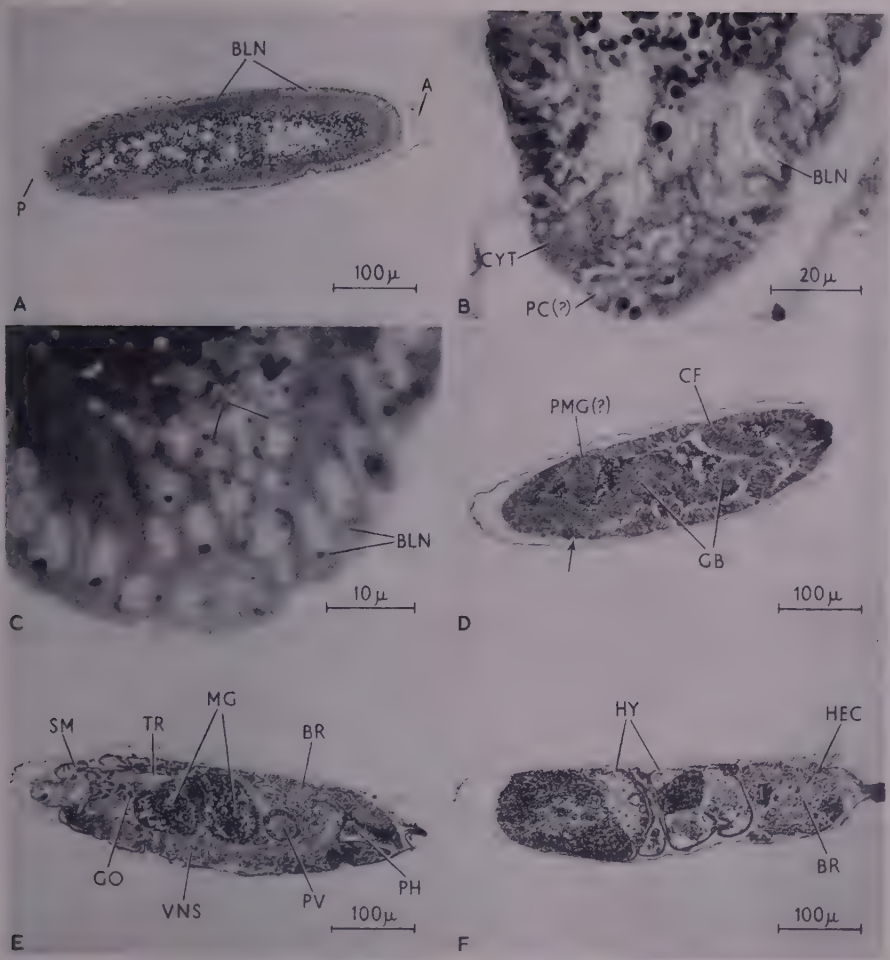

S. J. COUNCE *and* D. A. EDE

Plate 1

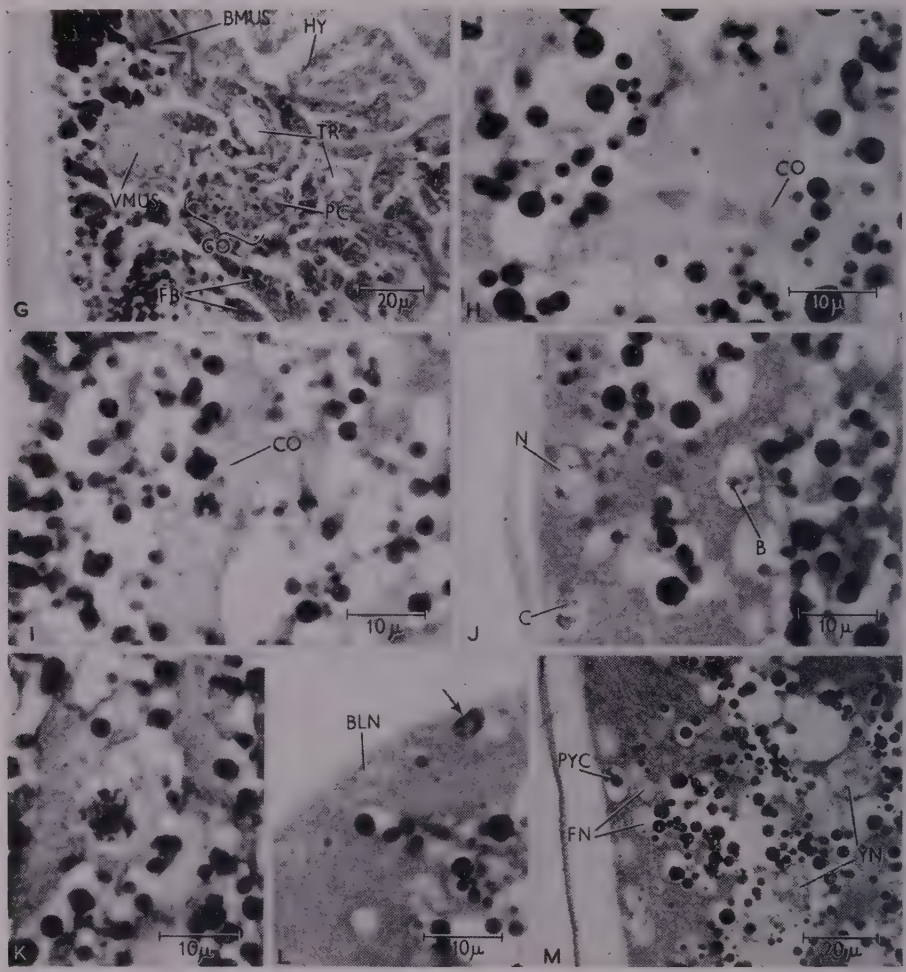

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Plate 2

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EXPLANATION OF PLATES

All figures are of *fs*^{nasA} embryos from mutant females mated to mutant males. Sections are 5–7 μ in thickness, and are stained with Heidenhain's iron haematoxylin. Photographs of entire sections are oriented so the anterior end is to the right.

Abbreviations: A, anterior; BLN, blastoderm nucleus; BMUS, somatic musculature; BR, brain; CF, cephalic furrow; CO, centriole; CYT, cytoplasm; FB, fat-body; FN, faintly staining nucleus; GB, germ-band; GO, gonad; HEC, head ectoderm; HY, hypodermis; MG, mid-gut; P, posterior; PC, pole cell; PH, pharynx; PMG, posterior mid-gut; PV, proventriculus; PYC, pycnotic nucleus; SM, segment; TR, trachea; VMUS, visceral musculature; VNS, ventral nervous system; YN, yolk nucleus.

PLATE 1

FIG. A. Frontal section of blastoderm stage showing differences in cleavage synchrony. Note size differences between nuclei in the anterior and middle regions. Cell-membranes are forming.

FIG. B. Frontal section of posterior tip, showing incomplete blastoderm formation. A broad band of cytoplasm containing yolk and nuclei extends to the tip while at the sides a cellular blastoderm has formed.

FIG. C. Migration of the pole cells through the blastoderm wall (3½ hours). All pole cells have migrated in this embryo.

FIG. D. Frontal section of gastrula (4½–5 hours). The shape of the germ-band has been distorted by deep lateral and ventral folds, and is exposed to the surface at one side (arrow).

FIG. E. Longitudinal section of 'segmented' embryo at end of development. Partial head involution has occurred. In the posterior region, segments have formed. The brain is exposed to the anterior surface. The posterior and anterior mid-gut rudiments have fused, and a well-developed proventriculus is present. Note the attenuated shape of the most posterior segment.

FIG. F. Longitudinal section of 'compartment' embryo at end of development. The posterior sections consist mainly of yolk and undifferentiated cells bordered by the hypodermis. Some nervous tissue and head ectoderm have formed in the anterior-most section.

PLATE 2

FIG. G. Tissue differentiation. The mesoderm has separated into its component elements. The visceral musculature has not become attached to the gut, and has clumped together. Some of the somatic muscle-cells have fused and elongated. A gonad has formed and contains a single germ-cell; the pycnotic cells in the gonad are probably derived from dying pole cells and mesodermal cells of the gonad.

FIG. H. 'Chromosomeless' spindle. The shape of the spindle and the clear area in its centre suggest the presence of some substance in the equatorial region.

FIG. I. Spindle organized by single centromere. The nuclear membrane is still intact at the opposite pole. Normal metaphase at right.

FIG. J. Stages in development of abnormal nuclei. N, normal; spindle forming; B, chromatin tending to clump; staining reaction not normal; C, abnormal; chromatin separated into lightly stained clump and darkly stained clump.

FIG. K. Abnormal polyploid metaphase, polar view. The fuzzy appearance of the chromosomes is apparent.

FIG. L. 'Cloudy' nucleus (arrow). Normal blastoderm nucleus at left.

FIG. M. Types of abnormal nuclei, including a typical pycnotic nucleus, and several faintly staining peripheral nuclei. At the right are abnormal yolk nuclei which have clumped together.

(Manuscript received 19 : ii : 57)

Correcting the Genetically Determined Sterility of W^vW^v Male Mice

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WITH ONE PLATE

INTRODUCTION

In the house-mouse the factor W^v (viable allele of the W-locus) has been described by Little & Cloudman (1937). The W^v factor, in homozygous condition, causes a typical macrocytic anaemia, a white coat with black eyes, and almost always sterility both in males and in females. The testes are small, with a thickened tunica albuginea. While the interstitial tissue is increased, the tubules contain few or no seminiferous cells. The pituitaries have been reported by Fekete, Little, & Cloudman (1941) as slightly enlarged and hyperaemic: 'the eosinophils (α-cells) seemed to predominate and enlarged vacuolated basophils were not present'. However, Veneroni (unpublished) did not find significant differences between the pituitary glands of six normal and five W^vW^v males, as regards the size of the gland, the frequency of the α-cells, and the morphological aspect of the β-cells.

Few experiments have been performed in order to correct pharmacologically the sterility of the W^vW^v male mouse (Bianchi & Manera, 1953). On the other hand, Russell & Russell (1948), by transplantation of normal ovaries, succeeded in obtaining pregnancies in W^vW^v females.

This report deals with pharmacological experiments performed to correct the male sterility of the genotype W^vW^v, on which Veneroni has briefly reported in a previous paper (1955). The experiments described in this paper have been undertaken on the basis of the histological studies just mentioned, of the results of Bianchi & Manera (1954) with administration of synthetic oestrogens (stilbenes), and of the findings of Veneroni (1954) in the x- (reticular) zone of the adrenal glands of W^vW^v males. The procedure was also based on the following considerations of a physiological nature:

(1) Spermatogenesis is thought to be the result of the combined action of f.s.h. (the follicle-stimulating hormone) and of i.c.s.h. (interstitial cell-stimulating hormone). The administration of such gonadotrophins and especially of f.s.h.

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might therefore stimulate the activity of the few germinal areas in the young animals.

(2) The administration of testosterone is believed to increase spermatogenesis (Gaarenstroom & de Jongh, 1946) and might produce quick effects in young animals in which spermatogenesis is normally still scanty. In the W^vW^v genotype, because of its general 'neotenic' condition, the consequences are expected to be greater. When high dosages of testosterone are used the pituitary secretion is at first cut down and spermatogenesis reduced, but later the testicular activity is increased as the accumulated hormones are released (Heckel, Rosso, & Kestel, 1951).

(3) The average dosages used were rather high because of the supposed low sensitivity of young peripheral tissues to the stimulating actions of the hormones. According to Hooker (1944) in the period just preceding puberty some tissues become sensitive to specially strong and specific stimuli. Since in the W^vW^v genotype the germinal hypoplasia of the testis becomes more extreme as the animals age, there is an additional reason for starting treatment early –at puberty or just after.

(4) The fructose concentration in the seminal vesicles of the W^vW^v males has been found almost as high as in normal animals (Manunta, Veneroni, Rossi, & Boggetti, unpublished), which might possibly suggest a normal androgen production and good nutritional conditions for viable spermatozoa.

(5) Though most W^vW^v mice are sterile (and this has been the case in all untreated W^vW^v males so far observed in our animal house), occasional exceptions have been reported (Little & Cloudman, 1937; Grüneberg, 1952). This suggests at least a potential restricted fertility.

MATERIAL AND METHODS

The twenty-four W^vW^v mice used in the experiment to be described were segregating from $W^v/+ \times W^v/+$ crosses of animals whose ancestors had been kindly turned over to us by Prof. H. Grüneberg, London. The genetical background of such animals was fairly homogeneous because of the inbreeding which occurred in the families selected for the experiments.

The drugs used for the treatments were: (1) 'Hemoantin' Richter, which is a gonadotropic hormone prepared from serum of pregnant mares; its action is chiefly follicle-stimulating (f.s.h.); (2) 'Perandren' Ciba, a solution of testosterone propionate in oil.

RESULTS

The treatments and results are summarized in Table 1. In almost all the animals, the treatments caused some change in testis histology; in a few cases this was only slight, in the majority it was obvious. In untreated young W^vW^v animals, the seminiferous tubules are usually lined by Sertoli cells with a few

small spermatogenetic areas. In old age the W^vW^v tubules show thicker walls (sometimes hyalinized), no spermatogenic activity, and the Sertoli cells are either vacuolated or altogether absent. This has been previously reported, chiefly by Fekete, Little, & Cloudman (1941) and by Preti (1952). The testes of treated young W^vW^v males appear macroscopically of unchanged size and weight (28–35 mg), but the histological picture is very close to normal as well as their consistency (the latter is quite poor in untreated W^vW^v animals). In fact, we have observed seminiferous tubules with stratified germinal epithelium, in which one can recognize spermatogonia, spermatocytes, spermatids, and spermatozoa. The five untreated W^vW^v animals, on the contrary, show the typical hypoplasia or aplasia of the germinal tissue accompanied by a relative hyperplasia of the interstitial cells, as found by previous authors. The histological appearance of treated and untreated testes is illustrated in the Plate, figs. A–D. Six out of twelve treated males succeeded in fertilizing normal females; these had normal pregnancies with well-developed foetuses or young carried to term.

Three out of four W^vW^v males which had one testis surgically removed at the beginning of the experiment showed some recovery of testicular structure as judged histologically. Two females mated to one of them became pregnant.

DISCUSSION

The results so far obtained show conclusively that the effect of the W^v gene on the seminiferous tubules can be corrected: the few small spermatogenic areas of young W^vW^v animals can be stimulated to increase their activity, spermatozoa make their appearance in great numbers, and fertilization may occur after copulation. This follows the administration of f.s.h. and of f.s.h. plus testosterone; it may also happen after the removal of one testis. The best results have been observed on animals which received intermediate amounts of hormones over a fairly long period (2 months). This holds especially for young animals, presumably because the condition of the old W^vW^v testes is less favourable. The results suggest three possible interpretations for the sterility-producing action of the W^v-factor in adult W^vW^v mice:

(1) The pituitary of W^vW^v male produces less f.s.h., or f.s.h. which is physiologically inactive. (The normal cytological picture, as far as the available data show, suggests that the possible abnormality of the pituitary can only be at a biochemical level);

(2) The W^vW^v testis is less sensitive to a normal amount of f.s.h.;

(3) Both these mechanisms may be involved.

The fact that the removal of one testis leads to a recovery of the other one might be tentatively interpreted as a result of compensatory hypertrophy associated with a relatively larger f.s.h. supply.

Since the difference between animals treated with testosterone and f.s.h. and those receiving f.s.h. only was possibly limited to a slight increase in the number

of the spermatozoa (Gaarenstroom & de Jongh hold that testosterone affects the maturation of the spermatozoa), it seems to support the hypothesis that the i.c.s.h. and testosterone situation in the W^vW^v animals is close to normal as suggested by previous results (histological studies and oestrogen treatment mentioned

TABLE 1

Treatments and results

Animal No.	*Age at the beginning of the experiments*	*Duration of treatment*	*F.S.H.*	*Testosterone*	*Histological result*[1]	*Offspring*[2]
	(days)	(days)	(i.u.)	(mg.)		
1	30	20	40	5	±	0
2	30	6	40	0	±	0
3	30	45	70	2·5	±	14
4	30	60	70	2·5	+	4
5	30	60	90	5	—	0
6	40	45	70	2·5	±	0
7	41	10	40	2·5	+	2
8	44	60	80	5	+	1
9	50	75	100	0	+	2
10	50	75	70	0	+	2
11	60	3	10	0	±	0
12	60	100	150	5	—	0
13	90	30	40	0	—	0
14	120	18	150	0	±	0
15	120	40	150	5	+	0
16*	90	—	0	0	—	0
17*	60	—	0	0	—	0
18*	45	—	0	0	—	0
19*	70	—	0	0	—	0
20*	45	—	0	0	—	0
21**	70	65	0	0	+	13
22**	90	40	0	0	—	0
23**	30	45	0	0	+	0
24**	30	45	40	5	+	0

* Untreated control.

** These animals had one testis removed surgically at the beginning of the experiments.

[1] *Key to histological results*: +, change to normality or very close to it; ±, fair recovery of the tubules; —, no change.

[2] Number of offspring when mated to fertile females during the period of treatment, plus one week after the end of it.

above; see chiefly Bianchi & Manera, 1954). Since even in the best cases the testis volume has not been changed, the effect of the drugs must be interpreted as an activation of the pre-existent spermatogenic areas, which are determined very early in the gonad development.

According to Mintz & Russell (1955) in *WW* mice there is a deficiency of the primordial sex cells. Preti (1952) and Borghese (1954) found that in the testes of *WW*, WW^v, and W^vW^v embryo and new-born mice the large cells are, respectively, almost absent, very poorly represented, and poorly represented. The large

cells are generally thought to be a sign of the division activity of the primordial spermatogonia.

In the light of the embryological and histological studies and of our own results, the sterility-producing action of the *W*-locus appears to be due to two mechanisms. One of them is a reduction of the primordial sex cells, the second one is endocrinological. It is unlikely that the first of these can be changed, but the second one is adjustable. When an appropriate correction is made to the endocrine defect, W^vW^v males may become fertile.

SUMMARY

In the house-mouse the genotype W^vW^v is usually sterile. Young male mice of this genotype have been treated with high dosages of f.s.h. (follicle-stimulating hormone) or with f.s.h. associated with testosterone propionate; in some, a unilateral orchidectomy was carried out. Most of the animals thus treated showed a significant recovery in spermatogenesis; some of them, mated to fertile females, had offspring. Untreated W^vW^v males of our colony have always proved sterile. The sterility-inducing action of the W^v-factor is believed to be accounted for by a deficiency in f.s.h. secretion and/or to a low sensitivity of the germinative tissue to f.s.h. in addition to a deficiency of the primordial sex cells. The treatments did not increase testis size: apparently they stimulated the few derivatives of the primordial sex cells without increasing their number. But this may be sufficient to abolish the sterility of the W^vW^v males.

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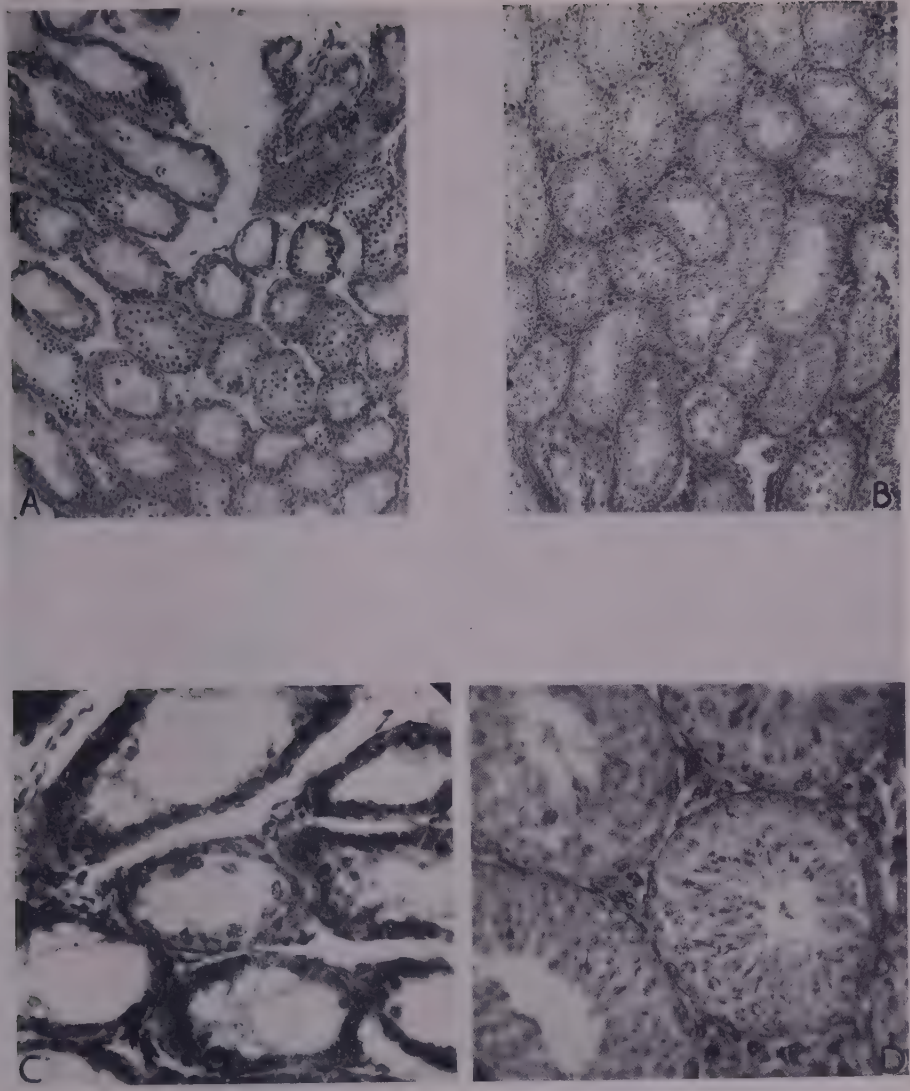

G. VENERONI *and* A. BIANCHI

Plate

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EXPLANATION OF PLATE

FIG. A. The usual aspect of the seminiferous tubules of the W^vW^v testis (animal No. 23, before unilateral orchidectomy). (Magnification *c.* ×70.)

FIG. B. W^vW^v testis after administration of f.s.h. and testosterone (animal No. 3). (Magnification *c.* ×70.)

FIG. C. Seminiferous tubules of W^vW^v testis surgically removed (animal No. 24). (Magnification *c.* ×250.)

FIG. D. Seminiferous tubules of the left testis in animal No. 24 treated with f.s.h. and testosterone. (Magnification *c.* ×250.)

(Manuscript received 8 : iii : 57)

Neuroglial Development and Myelination in the Spinal Cord of the Chick Embryo

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WITH FOUR PLATES

THE widespread occurrence of the demyelinating diseases of the central nervous system makes the study of the events that accompany normal myelin formation one of major significance in neuropathology. In the latter part of the last century the earlier stages in the development of the nervous system in embryos were studied in detail by many investigators, but since then, in spite of its evident importance, this aspect of embryogenesis has attracted little attention from embryologists and neurohistologists. In view of the advances now being made in the chemistry of the nervous system, the time seems opportune to return to the problem of myelinogenesis with the hope of bringing together some of the many important relevant observations in neural morphology and biochemistry.

In contrast to most earlier studies, the present one is restricted to some of the more outstanding features in the development of the neuroglia and myelin in the cervical spinal cord of the chicken embryo during the later stages of incubation. During this period the sequence of changes in the cellular population of the white matter in this region and the formation of myelin sheaths in its various funiculi proceed with remarkable rapidity. The speed of this development, taken together with its ready availability, the comparatively large size of the embryo, and its isolation during incubation from external biochemical influences, renders this species a particularly suitable one for the experimental morphological and chemical study of myelinogenesis.

MATERIALS AND METHODS

Fertile eggs from Sussex–Rhode Island crossed fowls were incubated at 38° C. They were turned daily and candled on the 5th day. Embryos were removed on each day from the 8th to the 21st days.

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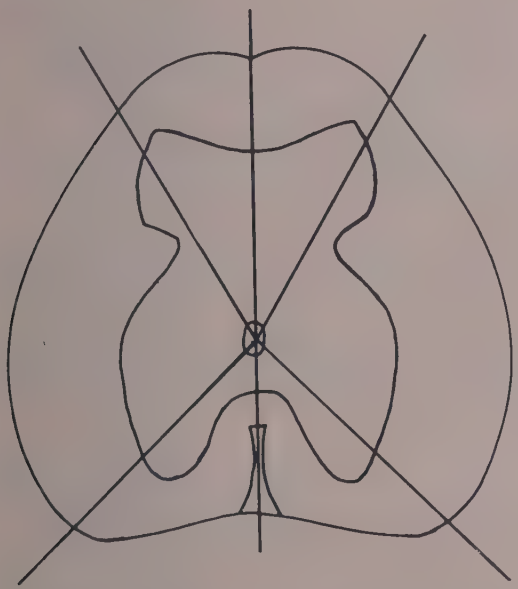
Histology. Fixation was carried out in 10 per cent. aqueous formol calcium (Baker, 1945) or in Zenker's fluid containing 5 per cent. glacial acetic acid. Adequate penetration was achieved by removing skin and soft tissues from the vertebral column in older embryos before fixation. After 24 hours blocks were cut from the upper cervical cord. Paraffin sections were prepared in the usual way to a uniform thickness of about 7 μ and stained with cresyl violet, luxol fast blue (Pearse, 1955), and by the Feulgen method. Additional sections, both transverse and longitudinal, were stained for axons by the method of Marsland, Erikson, & Glees (1954).



TEXT-FIG. 1. Diagram showing the method of arbitrary division of transverse sections of the spinal cord into 'anterior', 'lateral', and 'posterior' columns.

From a second series of embryos fixed in formol calcium, frozen sections were prepared. Some were mounted unstained in glycerine-jelly and examined by polarized light; others were stained with Sudan Black.

Other formalin fixed blocks from the same region were washed in tap-water, placed in 1 per cent. osmium tetroxide in phosphate buffer at pH 7·4 for 4 days, and then embedded in paraffin.

Cell counting procedure. For the purpose of enumerating the cells in the white matter, photomicrographs were made of the cresyl violet stained sections and enlarged to a final magnification of about 200 diameters. For each series of photographs a separate negative was made of the grid of an improved Neubauer haemocytometer under identical optical conditions, and this was superimposed

on the photomicrographs in a two-stage printing process in such a way that the lines did not obscure the details of the sections. This grid formed of squares each with sides of 50 μ greatly facilitated the counting.

The white matter of transverse sections of the cord was arbitrarily divided (see Text-fig. 1) into anterior, lateral, and posterior columns by two lines on each side which passed through the central canal at angles of 45° and 30° respectively to an antero-posterior line passing as nearly as possible through the anterior median fissure and the posterior median septum. The justification for this arbitrary division is that the first line approximately bisected the anterior horn and the second the posterior horn.

The area occupied by each column on the photomicrograph was measured with a planimeter and the number of nuclei, omitting those of endothelial and red cells, was counted square by square. The comparability of the daily counts was in some degree affected by a progressive change in the size of these nuclei: from the 9th to the 14th day of incubation there was a small reduction in their size as observed in longitudinal sections of the cord, but from that day to the end of incubation there was no further diminution. Such changes would lead to an underestimate of any increment in cell numbers that took place during the period of development studied (see Abercrombie, 1946). Both the nuclear counts and the area measurements were made independently by three of the authors and the means were calculated from their findings. Individual counts rarely differed by more than 10 per cent. from the mean. Actual areas of columns were calculated from the dimensions of the superimposed grid, and the densities of cells expressed as cells per $(100\ \mu)^2$.

The number of cells in the white matter undergoing mitosis was determined by examining, under oil immersion, sections that had been stained by the Feulgen method. Each author counted all cells in metaphase and anaphase in each of five sections, and the results (which corresponded closely) were expressed as the mean number per section. For these mitosis counts no attempt was made to enumerate those in each column separately.

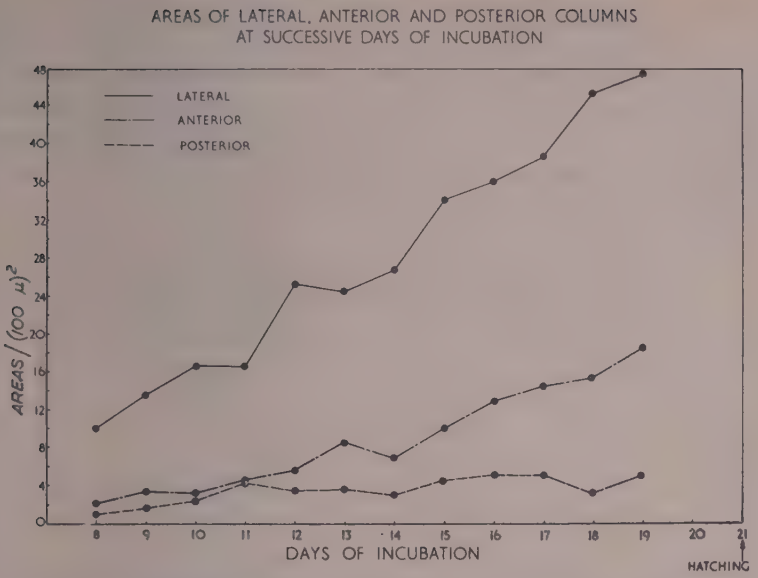
RESULTS

Growth in area of columns

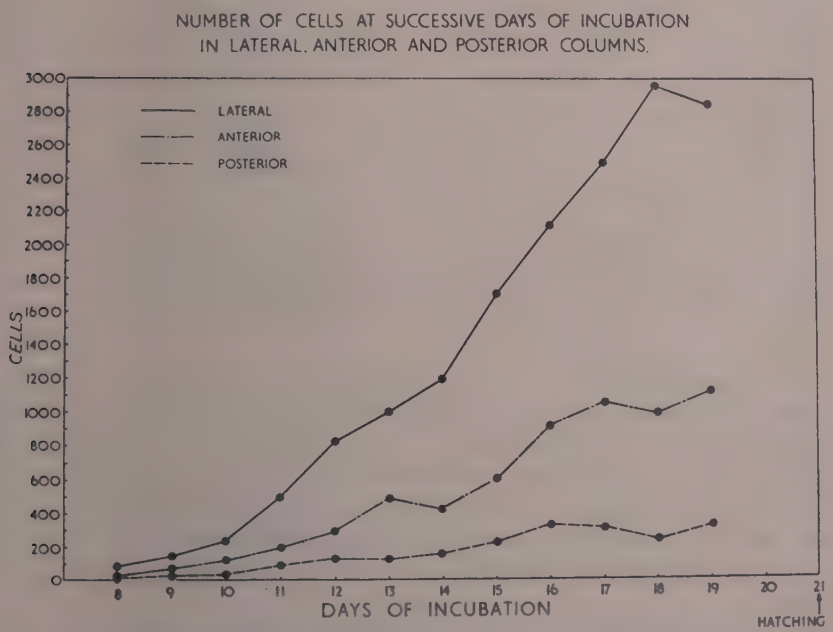
By the 8th day of incubation the white matter of the spinal cord at this cervical level forms approximately one-half of its entire cross-sectional area. The subsequent growth of these arbitrarily defined anterior, lateral, and posterior columns between the 8th and 19th days is shown in Text-fig. 2. During this period the anterior columns increase in area more than eight times, the lateral about five times, and the posterior about four times.

Neuroglial cells

Types of cell. Apart from vascular endothelial cells and erythrocytes, all the cells in the white matter appear to fall into two main types distinguishable from

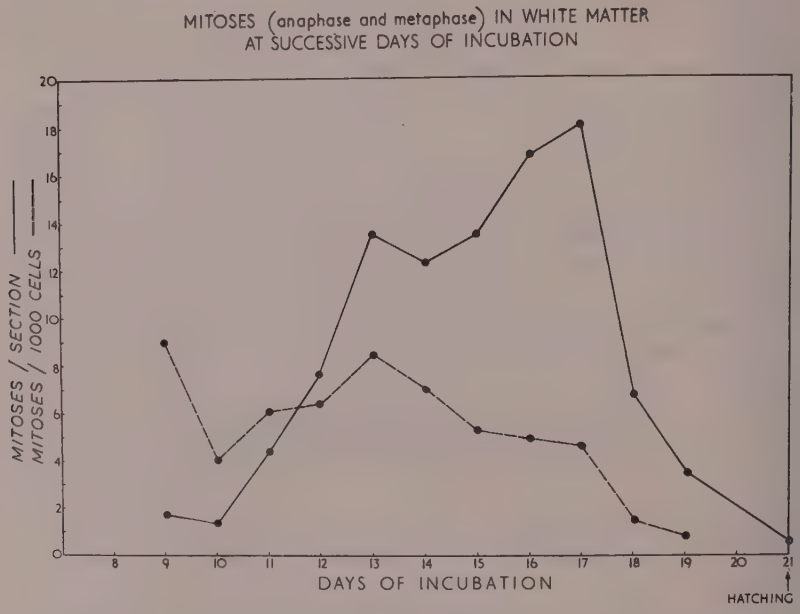


TEXT-FIG. 2. Growth in area of the anterior, lateral, and posterior columns at successive days of incubation from the 8th to the 19th day.



TEXT-FIG. 3. Numbers of cells in transverse sections of the spinal cord in the anterior, lateral, and posterior columns at successive days of incubation from the 8th to the 19th day.

one another by their nuclei. One type has a larger nucleus which is oval and vesicular, and contains several prominent nucleoli. Its chromatin stains less densely both with cresyl violet and by Feulgen's method than that of the smaller type. This latter is rounder, and its nucleoli tend to be obscured by its deeply staining chromatin. These two nuclear types (see Plate 1, fig. A) correspond to those described in pig embryos by Hardesty (1904) and as Types A and B in newborn rabbits by Spatz (1918).



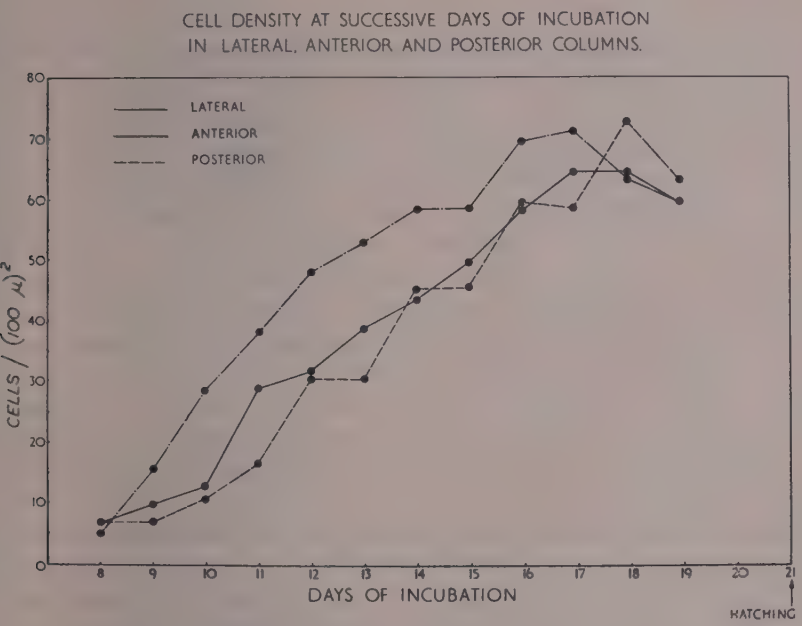
TEXT-FIG. 4. Mitoses in the white matter of the spinal cord at successive days of incubation from the 9th to the 21st day. (*a*) Total number in metaphase and anaphase per section. (*b*) Number per 1,000 neuroglial cells present.

Number of cells. On the 8th day the white matter is still relatively acellular, less than 100 nuclei being present in the section counted. Thereafter, the number increases steadily to reach more than 4,000 on the 18th day. Great though this recorded increment is, however, the true rise is almost certainly greater because of the diminution in the size of the neuroglial nuclei during this period. Text-fig. 3 depicts the participation of each of the three columns in this increase, together with the apparent diminution in the rate of increment between the 18th and 19th days.

Mitosis. The number of cells seen to be undergoing division reaches a maximum at the 17th day and falls suddenly thereafter (see Text-fig. 4) until, at the 21st day, only an occasional mitotic figure is seen. Expressed in terms of the total

number of glial cells present in the cord at that stage of development, mitosis is particularly frequent at about the 13th day.

Cell density. As can be seen from Text-fig. 5, the density of the glial cells increases at a similar and almost constant rate in all three columns, although that in the anterior columns precedes those in the others by about 2 days. The



TEXT-FIG. 5. Neuroglial cell density (number of cells per (100 μ)^2) in the anterior, lateral, and posterior columns at successive days from the 8th to the 19th day.

density reaches a maximum towards the end of the period studied. Only in the last day or two does it begin to fall; the anterior columns again precede the other two.

Distribution of glial cells in the white matter. The changing pattern of the distribution of the cells in the white matter is apparent from Plates 1–3, figs. B–F. By about the 11th day a pattern first becomes recognizable, for by this time the density of the cells has become notably greater in the anterior half of the cord. Their concentration is high in areas near the meninges and is maximal in comma-shaped strips that extend along either side of the anterior median fissure. By the 14th day this tendency towards a peripheral concentration is becoming less obvious, as the cells begin to increase in number in the more centrally situated parts of the anterior and lateral columns. During the last week of incubation the multiplying cells come to be distributed more evenly in all columns except for a relatively acellular zone adjacent to the grey matter, so that shortly

before hatching there are few signs left of the predominantly peripheral concentration of the neuroglial cells that was observable in the earlier stages. The much smaller posterior columns are also progressively colonized by neuroglial cells, though the process follows 2 or 3 days behind the comparable stages in the other two columns.

Development of myelin sheaths

No myelin sheaths are seen in the Sudan Black-stained preparations before the 11th day of incubation. In sections from some 11- and 12-day-old embryos, an occasional imperfectly formed sheath can be seen, and these always lie in the anterior columns in the marginal white matter adjacent to the meninges or the anterior median fissure. By the 13th day all the embryos show more myelin sheaths in these anterior marginal zones, and in some embryos sheaths are occasionally visible in those more centrally situated areas that lie near the grey matter. From the 13th to the 17th day the marginal distribution of sheaths in the anterior column spreads backwards into the contiguous marginal areas of the lateral columns. During this period myelin sheaths also appear in the more centrally placed white matter of these columns until, by the 18th day, their distribution is more or less uniform. In the posterior columns myelin sheaths are not seen until about the 14th day; they first appear in the centrally situated areas and extending thence towards the meninges come to be distributed more or less uniformly throughout these small columns by the 18th day.

When myelinated fibres become numerous, they are readily visible in sections stained with osmic acid and luxol fast blue, but of the various techniques available, examination by polarized light provides a particularly convenient method for illustrating the spread of myelination. In the transverse sections of the cord, myelinated fibres appear as bright areas, which with higher magnification can be seen as discrete Maltese crosses. From the 15th day the photomicrographs taken with polarized light (Plate 4, figs. G–J) corroborate the estimates of the spread of myelination reached with the various staining techniques.

A photomicrograph taken from the spinal cord on the 21st day—the day of hatching—shows that at that time the myelination is still progressing in all three columns (Plate 4, fig. J) although that in the posterior has not yet reached the advanced stage found in the anterior and lateral columns. It is worthy of note that during their study of the myelination in the cervical spinal cord of the kitten, Windle, Fish, & O'Donnell (1934) observed a similar progression posterolaterally from the anterior columns.

DISCUSSION

The source, or sources, of the many cells that are present in the white matter of the spinal cord of embryos in the later stages of development has been the subject of much discussion, the more important contributions to which have

been summarized by Cajal (1909*a*). Mainly on the basis of his own studies of early chicken embryos with metallic impregnation methods, Cajal expressed unequivocally the view of himself and others in the statement 'the neuroglial cells are all of ectodermal origin, for they are derived from elements of the primitive medullary canal'. During the period of development covered by the present study, which differs from that of Cajal in being chiefly concerned with the latter part of incubation, the cell population of the white matter in the cervical portion of the spinal cord increases at least fortyfold, and almost certainly even more so when its growth in length is also taken into consideration. No attempt has been made here to separate these neuroglial cells into the categories described on the basis of their nuclear morphology by Weigert (1895), Huber (1901), Hardesty (1904), and Spatz (1918). Cells of both the Types A and B of Spatz, as well as intermediary forms, are present in large numbers, though those of Type A greatly outnumber the others.

Cajal's belief that the neuroglial cells are of indigenous origin, and that their increase in number during development can be accounted for by local mitotic division, is supported by our observations. Spatz (1918) and Dekaban (1956) have also drawn attention to the occurrence of mitotic figures in the white matter of the spinal cord of embryo rabbits, but neither gave an account of any attempt to relate their frequency to the age of the embryo. In the present study it has been found that for much of the period investigated the proportion of cells in conspicuous metaphase and anaphase lay between 4 and 8 per 1,000 neuroglial cells in the white matter. Supposing that the average duration of these phases of division in neuroglial cells is similar to that of other types of cell for which it has been determined (Widner, Storer, & Lushbaugh, 1951), the increase in cell population can be accounted for by local multiplication; any assumption of a further immigration from elsewhere is thus unnecessary.

The patterns of the distribution of the neuroglial cells, on the one hand, and of the developing myelin sheaths, on the other, during the last week of development present a close resemblance. This similarity is well demonstrated by comparisons of the photomicrographs showing these cells after staining with cresyl violet and those displaying myelin taken with polarized light. Localized concentrations of the former are first recognizable in the white matter close to the anterior median fissure on about the 12th day; this area is also the site of appearance of the earliest myelin sheaths. During the ensuing few days the posterolateral spread of the concentrations of cells in the marginal areas adjacent to the meninges is shortly followed by a similar pattern in the development of myelin sheaths. This continuing close correlation persists into the last few days of incubation as first cells and then myelin sheaths come to be distributed more uniformly throughout the entire white matter of the three columns.

The most important question which arises from the foregoing comparison of neuroglial concentration and myelin sheath formation is the extent to which the two may be related functionally. The spatial distribution of both in the successive

stages of development studied is sufficiently close to suggest that myelination may take place when the density of the glial cells in the vicinity of the previously formed axons has attained a certain critical level. Although it seems likely that this observed correlation is more than fortuitous, it should not be overlooked that such an association of cells and sheaths may have resulted from the manner of growth of the white matter. For as Cajal (1909 *a*, *b*) pointed out, the increase in the white matter during development is brought about largely by the addition of new axons in those parts lying nearest the grey matter—a mode of growth that would tend to displace the earlier and presumably more mature fibres towards the periphery of the cord. But even if the similarity in the spatial distribution of the neuroglial cells and the myelin sheaths in the three columns can be accounted for on mechanical grounds by this mode of growth, it is difficult to believe that such an explanation would serve equally to cover the successive phases in the development of the cord when the process of myelination is viewed from a temporal as well as from a spatial standpoint.

SUMMARY

1. A study has been made of the development of the white matter in the spinal cord of the chicken embryo from the 8th day of incubation until hatching.

2. The increase in the neuroglial cells in the anterior, lateral, and posterior columns has been estimated from counts made on sections taken from embryos removed at daily intervals during this period. From counts of neuroglial cells undergoing mitosis in these sections, it would seem that the rise in their population could be accounted for by the multiplication of local cells.

3. Myelin sheaths appear in small numbers in the anterior column about the 12th day of incubation. From the 15th day onwards myelination proceeds actively, spreading backwards from the anterior columns in a general sweep laterally and posteriorly.

4. There seems to be a correlation in time between the rise in the density of neuroglial cells in different parts of the white matter and the onset of local myelination, the former appearing about 2 to 3 days before the latter.

ACKNOWLEDGEMENTS

One of us (R. T. W. R.) wishes to thank the Nuffield Foundation for their support as a Travelling Fellow. All the authors wish to thank Mr. J. F. C. Willder for the technical preparation of much of the material.

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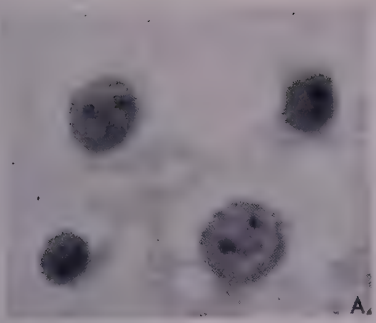
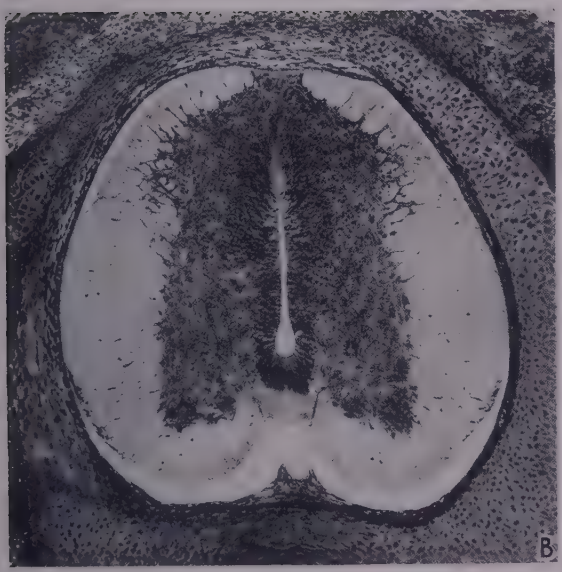
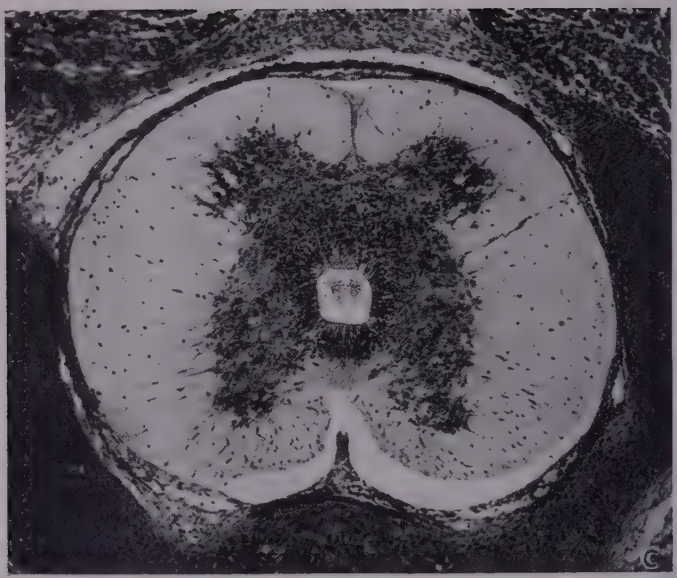




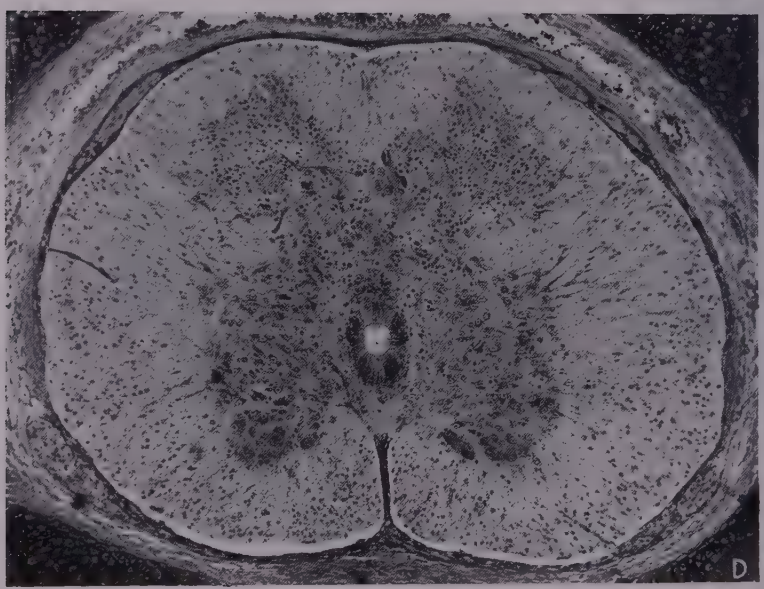
8 days

J. P. M. BENSTED, J. DOBBING, R. S. MORGAN,
R. T. W. REID, *and* G. P. WRIGHT

Plate 1



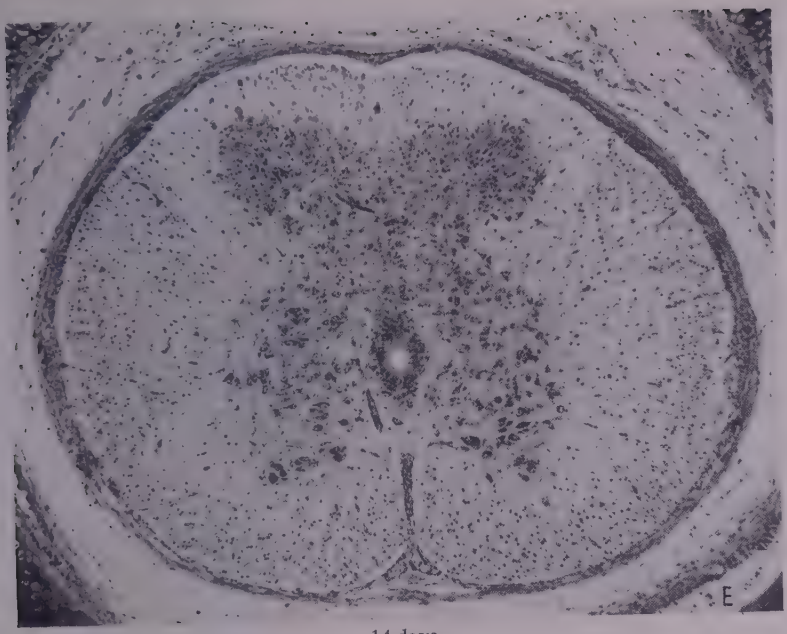
10 days



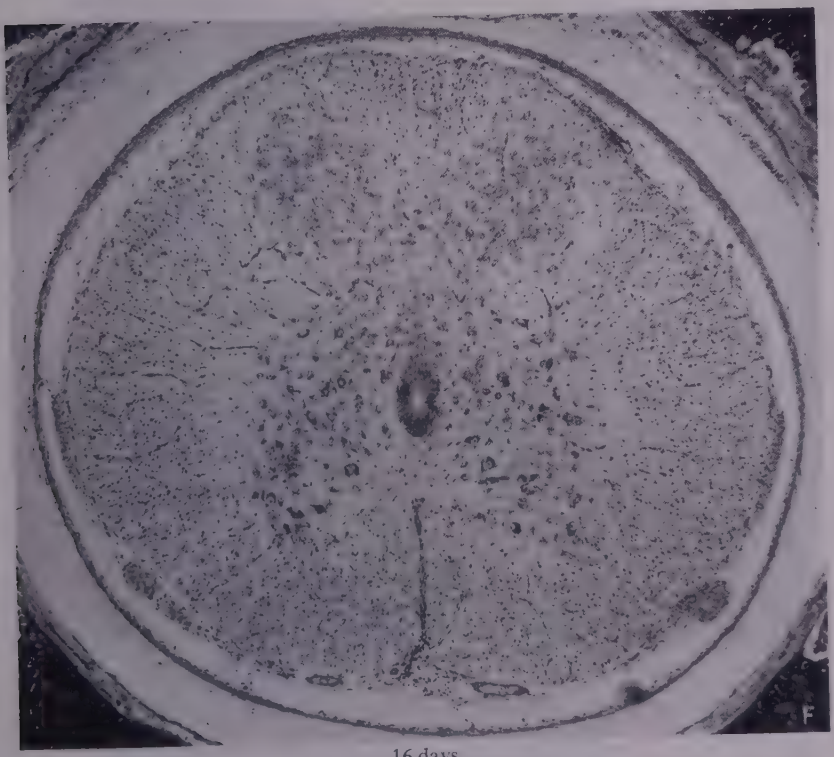
12 days

J. P. M. BENSTED, J. DOBBING, R. S. MORGAN, R. T. W. REID, *and* G. P. WRIGHT

Plate 2



14 days



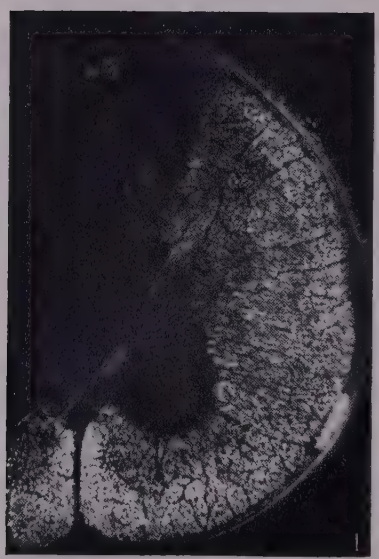
16 days

J. P. M. BENSTED, J. DOBBING, R. S. MORGAN, R. T. W. REID, *and* G. P. WRIGHT

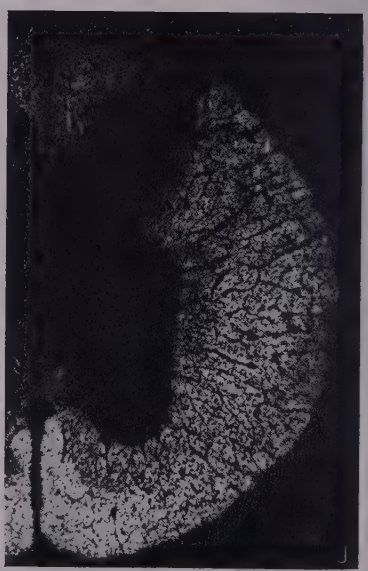
Plate 3

15 days

17 days



19 days



21 days

J. P. M. BENSTED, J. DOBBING, R. S. MORGAN,
R. T. W. REID, *and* G. P. WRIGHT

Plate 4

Cajal, S. Ramon Y. (1909*a*). *Histologie du système nerveux de l'homme et des vertèbres*, vol. i, p. 635. Paris: A. Maloine.

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EXPLANATION OF PLATES

Plates 1–3

Fig. A. Photomicrographs of four neuroglial cells, two with nuclei of Type A and two with those of Type B. Heidenhain's iron haematoxylin. ×1,000.

Figs. B–F. Photomicrographs of transverse sections of the cervical spinal cord on the 8th, 10th, 12th, 14th, and 16th days of incubation. Cresyl violet. ×100.

Plate 4

Figs. G–J. Photomicrographs taken with polarized light of unstained frozen sections of the cervical spinal cord on the 15th, 17th, 19th, and 21st days of incubation. ×70.

(Manuscript received 12:iii:57)

The Pluripotency of the Pharyngeal Primordium in Urodelan Neurulæ[1]

by TOKINDO S. OKADA[2]

From the Zoological Institute, College of Science, University of Kyoto

WITH ONE PLATE

INTRODUCTION

It was for a long time taken for granted that determination of the primordia of the endodermal organs of Amphibia occurs prior to gastrulation (cf. Holtfreter, 1938 *a*, *b*; Stableford, 1948). There have been, however, some data which seem to conflict with this statement. For instance, after the extirpation of some endodermal primordium or primordia from neurulae or more advanced embryos of urodeles, the remaining endoderm shows regulative development into an almost complete digestive tract, no deficiency of any endodermal organs being encountered (Balinsky, 1948; Okada, 1953; Mikami & Murakawa, 1954). Moreover, when a small endodermal fragment from a gastrula or neurula is transplanted into a heterotopic site of another embryo, its resulting differentiation is not *bedeutungsgemäss*, but *ortsgemäss* in many cases (Balinsky, 1938, 1948). Judging from these findings, we cannot help concluding that each endodermal primordium is not in a state of final determination even after gastrulation, although, of course, it may be possible that labile determination has already begun at a much earlier stage. This supposition is also supported by the results of the author's earlier experiments (Okada, 1955 *a*, *b*), in which the developmental potencies of each part of the endoderm were tested by means of explanting a small endodermal piece together with some mesodermal materials. These experimental results indicated that the development of a part of the endoderm of the neurula is not rigidly bound to its own prospective fate, since a very wide range of differentiation was found for each small piece. Moreover, the experiments showed that the resulting differentiation of endodermal pieces taken from the same source varies according to the mesodermal environments provided. However, a systematic analysis of the relationship between the endoderm and mesoderm was not carried out in these earlier works. It is still an open question

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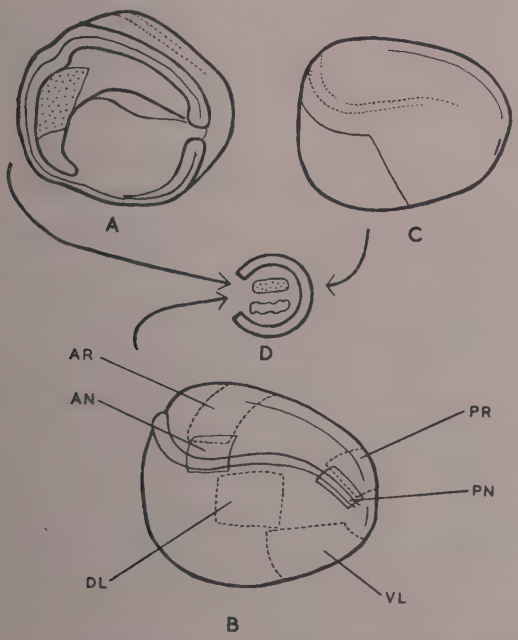
whether each region of mesoderm has a characteristic effect on the development of particular endodermal primordia.

The present experiments were carried out in order to examine whether the fate of endodermal fragments removed from one and the same source can be altered when they are explanted together with ecto- or endomesodermal materials taken from different regions of the neurula.

MATERIALS AND METHODS

As materials, neurulae of the Japanese newt *Triturus pyrrhogaster* were employed, and those of *Hynobius nebulosus* were also used less often. A major part of the present experiments consists of the homoioplastic explantation, within the epidermal envelope stripped off from the mesoderm-free belly region of a neurula, of the pharyngeal primordium plus ecto- or endomesodermal material cut out of various regions (see Text-fig. 1, A–D). In the control experiments, endoderm from the same source was explanted alone in the envelope. Besides, some xenoplastic combinations were carried out, i.e. the endoderm taken from *Hynobius* was combined with the ecto- or endomesodermal material of *Rana japonica*, and they were enveloped with an epidermal flap from either *Hynobius* or *Rana*.



TEXT-FIG. 1. Scheme of operation showing the locations and areas from which pharyngeal primordium (A), materials of ecto- and endomesoderm to be combined (B), and enveloping epidermis (C) were removed. See text for key to labels of mesodermal regions.

The pharyngeal primordium was cut out from the wall of the foregut, by reference to Nakamura & Tahara's 'Anlagenplan' for the endodermal area of Japanese urodelans (Nakamura & Tahara, 1954, and unpublished data). The materials to be combined with this endoderm were removed, as shown in Text-fig. 1, B, from (1) anterior one-fourth of the neural fold together with adjoining neural plate (designated as *AN* in the following description), (2) anterior one-fourth of archenteric roof (*AR*), (3) posterior one-fourth of neural fold together with adjoining neural plate (*PN*), (4) posterior one-fourth of archenteric roof (*PR*), (5) a dorsal half of the lateral plate in the middle trunk region (*DL*), and (6) a ventral half of the lateral plate in the caudal trunk region (*VL*). The endoderm and the envelope were removed exclusively from the earliest neurulae, in which the medullary folds were not elevated (stages 16 or 17 of Yo K. Okada & Ichikawa's standard series in the development of *Triturus pyrrhogaster*), while the ecto- or endomesodermal materials were taken from varied stages of the neurula (sts. 16–19).

In the neurulae used, the germ layers were so easily separable that each component of the explants was almost completely freed, by ordinary surgical operation, from an accidental contamination by cells of other germ layers.

Operations and cultivations were done in Holtfreter's solution which contained 0·1 per cent. sulphadiazine (Elkosin, CIBA) as bactericidal agent. All of the explants were kept at laboratory temperatures for 5–6 weeks, during which the control intact embryos reached sts. 53–55 of Yo K. Okada & Ichikawa's series.

RESULTS

A. *Control experiment: explantation of pharyngeal primordium alone within epidermal envelope*

All twenty-six explants when fixed appeared as shrivelled masses of irregular shape. The outer envelope was always atypical epidermis. In every explant of this experiment, the endoderm remained a random accumulation of heavily yolk-laden cells, showing no sign of differentiation towards any definable structure (Plate, fig. A). Although the aim of this control experiment was to see what tissue would be differentiated from the pharyngeal primordium in the absence of any mesodermal material, this could not be achieved by the present method. Presumably, under these experimental conditions, the development of the endodermal fragments is much retarded or rendered impossible.

B. *Combined explantation*

In contrast to the previous experiment most of the explants made of pharyngeal primordia together with ecto- or endomesodermal material had definitive endodermal structures as well as mesodermal and ectodermal ones. The endo-

dermal structures contained in these explants were generally so typical as to enable us to identify them as corresponding with the endodermal tissues of the normal embryo.

1. *Explantation of pharyngeal primordium together with anterior one-fourth of neural materials (group Ph plus AN)*

Twenty-five specimens were available, and they grew each into a large expanded irregular body with pigment cells on its surface. They were occasionally provided with balancers or eyes. Histological examination of serial sections of these explants showed the production of a typical pharynx (Plate, fig. B) in twenty-one cases. The wall of this pharynx was very thin and made of two-layered epithelium; further, it frequently communicated with the outside through the several branchial pouches produced. Around the pharynx there was a large amount of free mesenchyme. It is probable that all the mesenchymal cells contained in the present explants were derived from the neural crest contained within the compound *AN*-piece. This piece also differentiated cartilages and brain, accompanied sometimes by noses or eyes.

In the remaining four specimens of this group, the endoderm persisted as an amorphous mass of undifferentiated cells. These cases had a much smaller amount of mesenchyme than the other twenty-one cases.

2. *Explantation of pharyngeal primordium together with anterior one-fourth of archenteric roof (group Ph plus AR)*

Eighteen small swollen vesicles were microscopically examined. In this group the histogenesis of the endodermal component was generally not completed, each cell still retaining yolk-platelets at the time of fixation. However, there were nine cases in which the pharyngeal structure could be identified. In the explants of this group chorda and a few muscle-bundles were occasionally encountered. Free mesenchyme was also found in all cases, but its quantity was not so abundant as was found in the explants of *Ph plus AN*. These mesodermal tissues must be derived from the *AR* fragment added to the endoderm.

3. *Explantation of pharyngeal primordium together with posterior one-fourth of neural materials (group Ph plus PN)*

Twenty-one specimens were available, and all of them formed an elongated structure with a tail. The interior of the explant was filled with abundant free mesenchymal cells, amidst which muscles, chorda, and neural tube were present. The results indicate that the piece *PN*, which would comprise the whole primordia of the tail (cf. Nakamura, 1942; Chuang, 1947), underwent differentiation completely according to its prospective fate. The endoderm was always located amidst the mesenchyme, and differentiated mostly into typical pharyngeal structures, similar to those seen in the group *Ph plus AN*.

4. *Explantation of pharyngeal primordium together with posterior one-fourth of archenteric roof (group Ph plus PR)*

Each of twenty-three available specimens grew into a large expanded body of irregular shape, rarely with either a tiny tail-like process or an indefinable protuberance. Microscopical observation reveals that as to the endodermal differentiation pharynx was of universal occurrence except in two cases in which an indefinable endodermal mass was encountered. Chorda and muscles were found in all specimens of this group. These mesodermal and endodermal structures were always embedded within a large amount of free mesenchyme (Plate, fig. C).

In this group *Ph plus PR*, however, there was one special case, the interior of which was occupied by a large cell-free cavity lined with endothelial tissue, and the free mesenchymal cells were greatly decreased in amount, scattered within a narrow space between the wall of the cavity and the outer epidermis. The endoderm was situated in this narrow mesenchymal space. Its principal parts showed differentiation into pharynx, but one part, just in contact with the cavity-wall, had differentiated towards a gastric epithelium with several glandular follicles and towards oesophageal ciliated epithelium (Plate, fig. D).

5. *Explantation of pharyngeal primordium together with a dorsal half of the lateral plate (group Ph plus DL)*

All twenty-three available explants developed into large swollen vesicles, frequently possessing limbs. Microscopical examination indicates that a large coelomic cavity was always found in the interior of each explant; in the wall of this cavity the connective tissue-cells showed an endothelial arrangement. Blood-cells were frequently present within the cavity. In twenty-one cases the endoderm was immediately surrounded by the endothelial wall of the cavity, which corresponds to the splanchnic layer of the normal coelom, and was suspended by a mesenteric fold in the centre of the cavity. In such a location the endoderm took on a vesicular structure (except in three specimens in which a mere amorphous mass of undifferentiated endodermal cells was found). The wall of this endodermal vesicle was a one-layered epithelium composed of large cylindrical cells, as seen in Figs. E and F of the Plate. This characteristic permits us to consider the vesicle as an intestinal structure. Sometimes it was accompanied by liver or pancreas (Plate, fig. F).

In the explants of this group free mesenchyme was frequently lacking, or, if it occurred, was small in amount and found only in a restricted space between the epidermis and the outer or somatic wall of the coelomic cavity. Within such mesenchymal regions, pronephros and endoderm were encountered in 8 and 4 cases respectively. The endoderm which happened to be placed in these regions showed a typical differentiation into pharynx, in contrast with the differentiation into intestine of the endoderm located in the cavity. Two out of four cases with pharynx had an intestinal vesicle in the cavity also; and these endodermal struc-

tures were connected to each other by the intervention of a gastric epithelium with glandular follicles and the ciliated epithelium of the oesophagus.

6. *Explantation of pharyngeal primordium together with a ventral half of lateral plate (group Ph plus VL)*

Twenty-seven swollen vesicles were histologically examined. The resulting differentiations in the explants of this group were essentially similar to those described in the group *Ph plus DL* just above, with the exception of the absence of limb and pronephros in this group.

TABLE 1

Endodermal and mesodermal differentiations in the combination explants

For key to designations of material combined with pharyngeal primordium, see p. 440.

Material combined with pharynx	*AN*	*AR*	*PN*	*PR*	*DL*	*VL*	*Rana AN*	*Rana VL*
No. of available cases	25 (4*)	18 (9)	21 (3)	23 (2)	23 (3)	27 (3)	10 (6)	4 (2)
No. of explants showing the differentiations specified below:								
Pharynx	21	9	18	21†	4†	5†	4	0†
Oesophagus	0	0	0	1	2	5	0	0
Stomach	0	0	0	1	2	6	0	0
Intestine	0	0	0	0	18	21	0	2
Liver	0	0	0	0	9	8	0	0
Pancreas	0	0	0	0	2	2	0	1
Chorda	0	7	15	21	0	0	0	0
Muscle	0	12	17	23	7	0	0	0
Pronephros	0	0	0	0	8	0	0	0
Mesenchyme	24	16	21	23	13	11	9	1
Cavity	1	3	0	1	23	27	0	4
Blood	0	0	0	0	15	20	0	3

* The figures in parentheses indicate the number of specimens in which no definable endodermal structure was contained.

† In the groups containing *PR*, *DL*, *VL*, or *Rana VL*, a number of different endodermal structures have been formed within each of one or more explants. (NOTE: Since no difference was found between the results on *Triturus* and those on *Hynobius*, both data have been consolidated in this Table.)

The principal product from the pharyngeal primordium was an intestinal vesicle, as in the previous group. It was found in twenty-one cases. Liver and pancreas sometimes appeared together with it. Six out of these twenty-one cases with intestinal vesicles had also pharyngeal, oesophageal, or gastric tissues besides. Of these endodermal derivatives, structures of the gut anterior to the stomach were located within or adjacent to the narrow mesenchymal area between the central cavity and outer epidermis.

In another three cases, a pharynx was found as the only endodermal structure,

and this was within the mesenchymal area. In the remaining three cases the endoderm persisted as an amorphous mass of undifferentiated cells.

It is evident therefore, from the data reported for the groups *Ph plus DL* and *Ph plus VL*, that *bedeutungsfremde* differentiation towards posterior structures of the gut can occur when the pharyngeal primordium is explanted together with lateral plate mesoderm.

C. *Xenoplastic explantation*

In xenoplastic explantations, two groups of experiments were carried out. Although only in a few cases did the explants survive long enough, the results agreed well with the data obtained from homoioplastic explantations.

1. *Explantation of pharyngeal primordium of* Hynobius *together with anterior one-fourth of neural materials of* Rana *(group Ph plus Rana AN)*

Ten cases were available in this group. In serial sections the cells of *Rana* are easily distinguishable from those of *Hynobius* by their smaller size of nucleus and cytoplasm. In 4 out of these 10 cases, differentiation of pharynx took place and the latter consisted of cells of *Hynobius* origin. Around the pharynx there were many mesenchymal cells of *Rana* origin. Brain and cartilages of *Rana* were also found amidst the mesenchyme. In the other six cases the endoderm remained an amorphous mass of heavily yolk-laden cells of *Hynobius*, although the resulting differentiation from *Rana* was similar to that of the four cases described above. Symptoms of degeneration in the undifferentiated endodermal cells were observed in 2 of these 6 specimens.

2. *Explantation of pharyngeal primordium of* Hynobius *together with a ventral half of lateral plate mesoderm of* Rana *(group Ph plus Rana VL)*

Six cases survived for 30 days or longer after operation. But in one of these the endoderm was extruded out of the enveloping epidermis during the course of cultivation, and degenerated. Moreover, there was another case in which the endodermal component degenerated within the mesodermal environment of *Rana*, leaving only cell debris. Therefore, only four cases were really available in this group. The endoderm of *Hynobius* was located in the centre of a coelomic cavity, being surrounded by thin endothelial tissue of *Rana* origin. In two cases this endoderm showed a clear differentiation into an intestinal vesicle (Plate, fig. G), while the other two cases had only an amorphous accumulation of undifferentiated endodermal cells. A small pancreatic structure was found in 1 of the 2 cases with intestine.

From these results of xenoplastic explantations, it can be stated that the lateral plate of *Rana* has also a faculty, although it is less effective than that of Urodelans, of switching the fate of the pharyngeal primordium of urodelan neurulae to the production of an intestinal vesicle.

DISCUSSION

In the present experiments a great diversity of endodermal structures, including pharyngeal, oesophageal, gastric, intestinal, and accessory glandular tissues, was found to develop from the pharyngeal primordium, when explanted combined with ecto- or endomesodermal material, as summarized in Table 1. The question may arise whether all these endodermal structures are really derived from the explanted endoderm removed from the one source, i.e. from the prospective pharyngeal area. Is there any possibility that the ecto- or endomesoderm included, combined with it accidentally, some endodermal cells, and the latter developed into such endodermal structures as are found within the combined explants? The answer is no, because, as described above, removal of pure ecto- or endomesodermal pieces free from contamination with the cells of the other germ layers could be simply carried out in my material. Such a success in the operation can be demonstrated also by the fact that, when these ecto- or endomesodermal materials were explanted alone within epidermal vesicles, no trace of endodermal cells was found (the author's unpublished data). Thus we can conclude with considerable certainty that all of the endodermal structures found within the combined explants have been derived exclusively from the pharyngeal primordium. Further conclusive evidence of this is afforded by the results of the xenoplastic explantations, in which pharyngeal or intestinal structures were differentiated from the pharyngeal primordium of *Hynobius*, the materials of *Rana* combined with it never taking part in the production of the endoderm. Consequently, we may safely state that the pharyngeal primordium in the early neurula stage is not yet so rigidly determined as to perform differentiation only to its prospective fate, but possesses pluripotency to develop into various endodermal structures.

According to the present results there exists a large difference between the groups including lateral plate mesoderm (pharyngeal primordium combined with *DL*, *VL*, or *Rana VL*) and the other groups (pharyngeal primordium combined with *AN*, *AR*, *PN*, *PR*, or *Rana AN*) in respect to the endodermal structures differentiated. In the former groups, intestine is a principal structure and pharynx, oesophagus, and stomach are rather subordinate. On the other hand, in the latter groups, pharynx is of common occurrence regardless of the source from which the combined piece of ecto- or endomesoderm originated (cf. Table). But, looking through the data presented in this paper, we can find the following general rule, which governs all the groups of the present experiments: the pharyngeal differentiation occurs always amidst free mesenchyme, whereas the intestinal structures are found coated with an endothelial covering. And the oesophageal or gastric differentiation always takes place in a part of the endoderm where both kinds of mesodermal tissues, free-mesenchyme and endothelium, are present together. As far as the present results go, the presence or absence of the other ecto- or mesodermal structures, e.g. neural organs, chorda,

muscle, pronephros, limb, &c., seem to have no direct bearing on the endodermal differentiation. Accordingly, one may assume that the developmental fate of pharyngeal endoderm can be greatly altered in accordance with the sort of mesodermal tissues which are developing around the endoderm.

This statement seems to be in conflict with the conclusion of Holtfreter (1938 *a*, *b*), who claimed that every endodermal primordium is materially determined already in the blastula or earlier stage, so as to carry out further differentiation only into its prospective fate. But, from the viewpoint of the present author, Holtfreter's conclusion seems to be untenable, since it was drawn merely from the explantation of endodermal fragments alone, without any attempt to explant it together with various ecto- or endomesodermal materials.

There is a point in Holtfreter's experimental results which led him to the conclusion mentioned above to which we should pay special attention. He reported that a piece of gastrula endoderm of various European amphibian species, when explanted alone, can undergo self-differentiation into an endodermal tissue corresponding to its prospective fate. On the other hand, with Japanese urodeles we failed to obtain any definable differentiation from the explanted endodermal piece in the absence of a mesodermal environment, as was shown in the control experiments of the present work. However, such a negative result does not necessarily indicate a complete lack of self-differentiating capacity of the endodermal primordia in *Hynobius* and *Triturus* neurulae. The present author (Okada, 1951) has also obtained a self-differentiation of endoderm of *Hynobius* neurula in experiments in which the whole endodermal material, instead of a small piece, was removed and cultivated *in vitro*. In such a large explant, consisting of endodermal cells only, the region of the foregut could differentiate, though not quite typically, towards pharyngeal tissue, while in the remaining part only intestine-like tissue was encountered. The same phenomena was reported by Mangold (1936) in the *Triturus* neurula and by Stableford (1948) in the *Amblystoma* gastrula or blastula.

Judging from these experimental results, we cannot help admitting that the pharyngeal primordium in the gastrula stage, or a little earlier, has already a tendency to differentiate into pharynx; but the appearance of such a tendency does not mean that this primordium is irrevocably determined prior to gastrulation, because even in as late a stage as the neurula the future fate of the pharyngeal primordium is widely changeable by the influence of adjacent mesoderm, as is clearly shown in the present results. Under these circumstances, the supposition may be permitted that both a factor residing in the pharyngeal primordium itself, and influences from the mesenchyme, or from the precursor of mesenchyme, which comes into contact with this primordium in embryogenesis, are involved in the process leading to the final determination of the pharynx, although it is still unsettled in what stage the determination becomes final.

SUMMARY

1. Explantation experiments of pharyngeal endoderm, alone or together with various ecto- or endomesodermal materials, within epidermal envelopes were performed, using the neurulae of *Triturus pyrrhogaster* and *Hynobius nebulosus* as materials.

2. The piece of endoderm, when explanted alone within the envelope, did not differentiate into any definable structure, persisting as an amorphous mass of undifferentiated endodermal cells, even after a prolonged cultivation as long as 30–40 days. Typically differentiated endodermal structures were found only in the explants in which the endodermal piece was combined with the ecto- or endomesodermal materials.

3. When the pharyngeal primordium was explanted together with the material of either neural fold or archenteric roof, irrespective of the regions from which these materials were removed, the production of pharynx always occurred.

4. When combined with lateral plate mesoderm, the pharyngeal primordium generally developed into an intestinal vesicle. Oesophagus, stomach, liver, or pancreas was occasionally found in these combination explants.

5. Some xenoplastic explantations, in which the pharyngeal rudiment of *Hynobius* was combined with ecto- or endomesodermal material from the neurula of *Rana japonica*, were also carried out. *Hynobius* endoderm showed differentiation into pharynx when combined with anterior neural material of *Rana*, but it produced an intestinal vesicle when lateral plate of *Rana* was included.

6. In all the available specimens of the present experiments it was a rule that pharyngeal differentiation occurred within free mesenchyme, while intestinal structures were found to be surrounded by endothelial tissue.

7. On the basis of above data it is concluded that the pharyngeal primordium is still pluripotent in the early neurula stage, and its future fate may be varied, depending mainly upon the sort of connective tissue which is present around it.

ACKNOWLEDGEMENT

I should like to thank Prof. Dr. M. Ichikawa for his encouragement and valuable criticisms during the course of this work. I am also deeply indebted to Prof. Waddington, Sc.D., F.R.S., for reading the manuscript.

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EXPLANATION OF PLATE

FIG. A. Section through a small shrivelled mass of a control experiment with *Triturus*. Amorphous mass of undifferentiated endodermal cells and enveloping atypical epidermis.

FIG. B. Section through an explant of *Ph plus AN, Triturus*. Pharynx (*PH*), cartilage (*CR*), and brain (*B*) are present within mesenchyme.

FIG. C. Section through an explant of *Ph plus PR, Triturus*. Pharynx (*PH*) is formed near a large chorda (*CH*).

FIG. D. Section through another explant of *Ph plus PR, Triturus* with a small amount of mesenchyme. Gastric epithelium with glandular follicles (*G*) and ciliated epithelium of oesophagus (*OS*) are seen near an endothelial lining.

FIG. E. Section through a vesicular explant of *Ph plus DL, Triturus*, showing a large intestinal vesicle (*IT*) in the coelmic cavity.

FIG. F. Section through an explant of *Ph plus DL, Hynobius*. Intestinal vesicle (*IT*) and liver (*L*) in the cavity, and pronephros (*NP*) in the mesenchymal area.

FIG. G. Section through a xenoplastic explant of *Ph plus Rana VL*, showing intestinal vesicle (*IT*) and pancreatic structure (*PC*).

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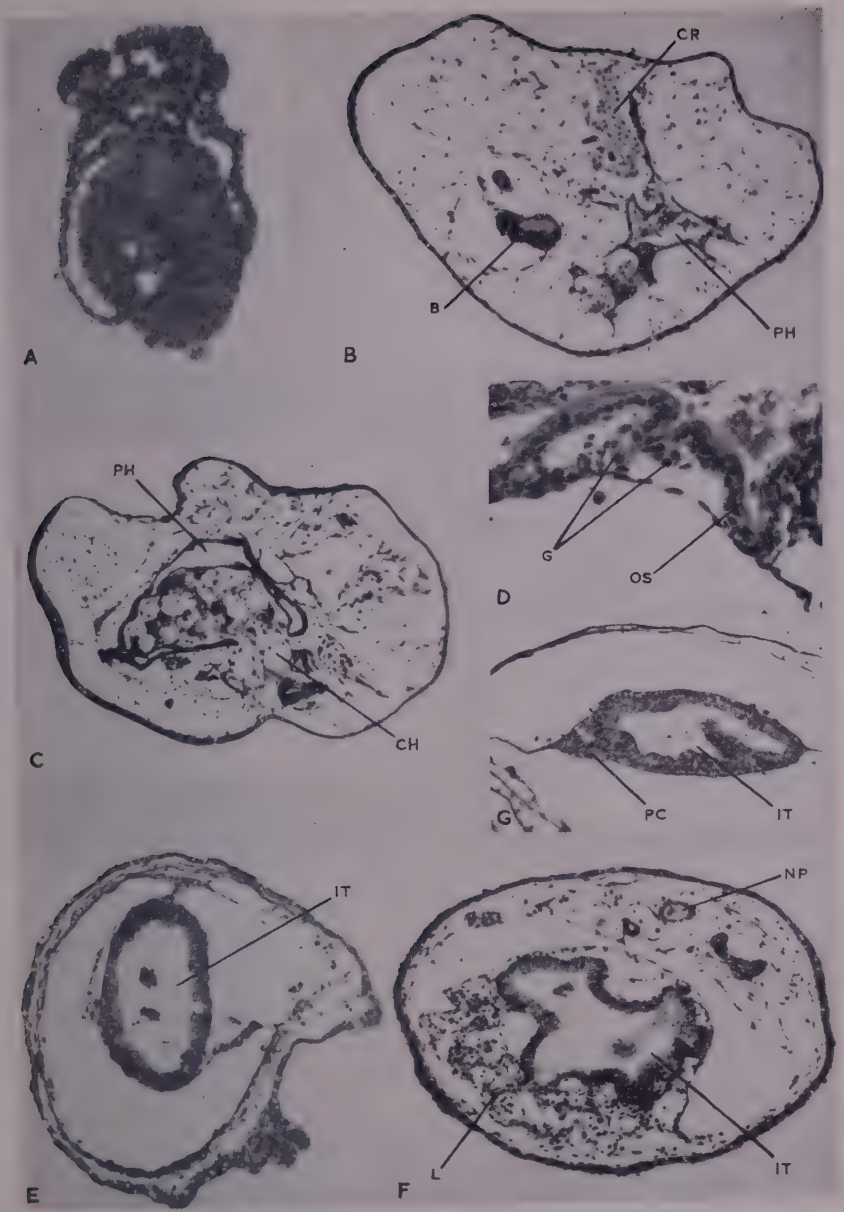

T. S. OKADA

Plate

Complete Heterospermic Androgenesis in Silkworms as a Means for Experimental Analysis of the Nucleus-Cytoplasm Problem[1]

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WITH TWO PLATES

In the course of the last 70 years many outstanding biologists have tried to obtain so-called heterospermic merogons, or androgenetic hybrids, developing as a result of interaction between the cytoplasm of one (maternal) and the nuclear material of another (paternal) species. The successful achievement of this experiment was considered the *experimentum crucis* for the solution of certain fundamental questions of heredity and development pertaining to the so-called nucleus-cytoplasm problem. The most important of these questions was that of the cytoplasmic *versus* nuclear localization of the physical elements which condition the specific hereditary characters of parental forms and their transmission through successive generations.

The problems of merogony, androgenesis, and related questions raised in the pioneer works of Th. Boveri (1888–95), Oscar & Richard Hertwig (1887), Y. Delage (1898, 1899), and T. H. Morgan (1895) still attract the attention of biologists today. A review of the problem up to 1948 is given in the article by Astaurov (1948). Among the many experimental zoologists who devoted much ingenuity and labour to this field, mention should be made of H. Spemann, Fr. Baltzer, R. Goldschmidt, E. Godlewski, C. Herbst, Günter and Paula Hertwig, J. Hämmerling, J. Harder, G. Fankhauser, A. Dalcq, E. Hadorn, H. Curry, S. Hörstadius, L. von Ubisch, C. T. Kaylor, K. R. Porter, B. C. Moore, H. C. Dalton, and others.

However, in spite of numerous attempts, adult heterogenous-androgenetic animals have not yet, as far as we know, been obtained. In the most successful cases the investigators were able to obtain either haploid androgenetic embryos,

[1] Paper read at the International Congress of Developmental Biology (23–26 July, Brown University, Providence, U.S.A.); see Astaurov & Ostriakova-Varshaver (1957).

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[3] We regret to report the death of Dr. Ostriakova-Varshaver on 22 September 1957.

[J. Embryol. exp. Morph. Vol. 5, Part 4, pp. 449-462, December 1957]

which usually perished in the earliest stages of development when the specific characters of parental species were almost indistinguishable, or haploid androgenetic tissues which can survive only as grafts on normal embryos of the parental species.

The overwhelming majority of these experiments were made by zoologists on sea-urchins and amphibians, and in the new hand-book *Analysis of Development* (Willier, Weiss, & Hamburger, 1955), for example, only the results obtained on these animals are mentioned and discussed.

An advance in the solution of the problem has meanwhile been made by means of experiments on insects. A complete dispermic diploid androgenesis in silkworm was obtained almost simultaneously, though independently and by slightly different methods, by the Japanese geneticist Hasimoto (1934) and by Astaurov (1936, 1937); and a complete haploid androgenesis in the parasitic wasp *Habrobracon juglandis* was obtained by Anna Whiting (1946 *a*, *b*).

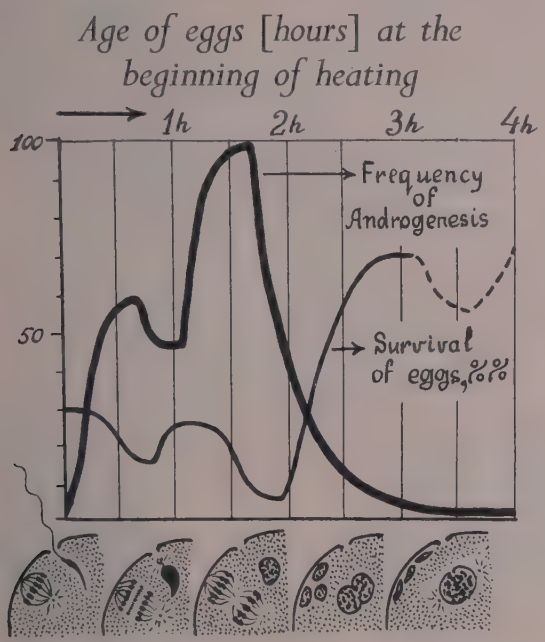
As the biology of the silkworm has been thoroughly investigated, its rearing is easy and its propagation fast, this organism is a very convenient object for studying difficult problems on the borderline of experimental embryology and cytogenetics, and especially problems of artificial parthenogenesis and androgenesis (Astaurov, 1957). In our present communication we hope to show how, using the domesticated silkworm *Bombyx mori* L. and its near relative the wild species *B. (Theophila) mandarina* (Moore), it became possible to achieve the long-standing aim of complete interspecific androgenesis.

The wild silkworm living in South-East Asia is so different from the domesticated silkworm *B. mori* L. that Moore, in his first description of this insect in 1872, considered it as belonging to another genus and named it *Theophila mandarina*, under which name it is sometimes referred to even today. Nevertheless, it is undoubtedly very similar to the ancestral species from which *B. mori* originated and should therefore be put in the genus *Bombyx* (Sasaki, 1898; Conte, 1911; Bobrov, 1936; Ishihara, 1943). It has even been suggested (Sasaki, Conte) that it should be considered only a subspecies or a variety of *B. mori*, though this view can hardly be accepted.

It seems that any answer to the question as to whether we are dealing here with two good species or with incipient ones only will always be to some extent uncertain. The question is made difficult by the fact that one of the two forms is greatly changed as a result of artificial selection in conditions of domestication. The only indisputable fact is that, in spite of their possessing many similar features determined by close relationship, they are so different in almost all morpho-physiological, biological, and ecological characters that at first glance one would, without the slightest hesitation, classify them as different species and even, as we have seen, as different genera.

B. mandarina has a haploid chromosome number of 27, while *B. mori* has 28 (Kawaguchi, 1928). *Mandarina* caterpillars are of dark, protective so-called *Mauricaud* marking, while the caterpillars of various *mori* races have different

markings, among which *Mauricaud* caused by the dominant factor p^M, is also met with, although this occurs relatively seldom and mostly in primitive races. *Mandarina* moths are dark, greyish-brown, while those of *mori* are white or cream-coloured. Brown coloration in *B. mori* caused by the dominant factor *Wm* (*Wild melanism*) occurs very seldom. In *mandarina* sexual dimorphism is sharply expressed, while but slightly in *mori*. *Mandarina* cocoons are very small and poor in silk content. They are pointed, light greenish-yellow, and enclosed



TEXT-FIG. 1. Change in susceptibility to heat shock and in capacity to undergo thermal androgenesis in the eggs of *B. mori* during the course of maturation and fertilization. Thin line: percentage of hatched larvae (reciprocal of susceptibility); thick line: percentage of androgenetic larvae (the maximum yield taken as 100 per cent.).

in a filmy coat lining. *B. mori* has completely lost some of the wild instincts well developed in *mandarina*: its caterpillars are sluggish and would rather die than creep in search of food; its moths do not fly. The Ussuri *mandarina* race used in these experiments has a rare and peculiar feature, which is not met with in any of the *mori* races: its hibernating generation has two diapauses; besides the embryonic one it possesses a pupal diapause.

In addition to this, the chorion of hibernating *mandarina* eggs is covered with a spongy layer of wax-like substance which makes the egg-shell opaque. This does not occur in *mori*.

Crossing between these two forms is possible and gives fertile hybrids, but

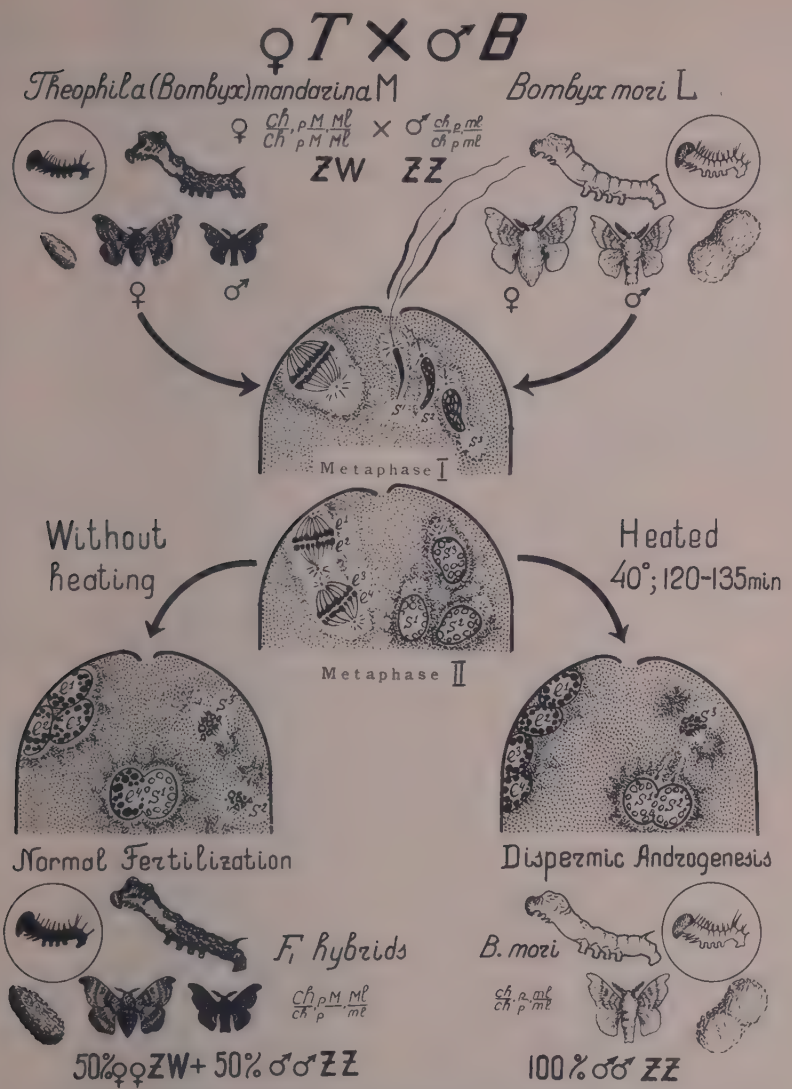
because of the great differences in body-size and sexual instincts of the moths, crossing is difficult and requires the aid of the experimenter and a special technique. An attempt to obtain a heterospermic androgenesis may be made in both reciprocal crosses. In Plate 2, fig. C, photographs of two copulas of moths mated in reciprocal combinations are given.

To prevent a female nucleus from taking part in the process of fertilization and to obtain dispermic androgenetic development a method of heat treatment was used. Numerous experiments carried out by Astaurov, Ostriakova-Varshaver, & Strunnikov (1957), the results of which are given diagrammatically in Text-fig. 1, showed that the susceptibility of a freshly inseminated egg to heat changes greatly and regularly in the course of maturation and fertilization. These experiments and a cytological investigation carried out by Ostriakova-Varshaver (1952, 1957 *a*, *b*) established that androgenetic individuals arise in *B. mori* most frequently when heat treatment (usually 40° C. for 135 minutes) begins approximately 90 minutes after laying, immediately before the most sensitive stage of the process of maturation—metaphase of the 2nd meiotic division. Heating to 40° C. at this time destroys the spindles of the second maturation division which are situated near the egg surface. As a result, all the products of the division of the oocyte nucleus remain in the egg periphery far from the several sperm nuclei (potential male pronuclei) waiting for karyogamy in the depths of the ooplasm (Ostriakova-Varshaver, 1952, 1957*a*). For the male haploid nuclei nothing remains but to fuse with each other, thus forming by means of a peculiar male self-fertilization a diploid male synkaryon of an androgenetic oosperm (Ostriakova-Varshaver, 1957*b*).

The technique of experiment in the two reciprocal crosses is a little different and we shall consider them separately.

1. Androgenesis after the cross ♀ *B. (Theophila) mandarina* × ♂ *B. mori* [*T* × *B* type] (*mandarina* plasm + *mori* nucleus, see scheme in Text-fig. 2).

For identification of heterospermic androgenetic individuals in the *T* × *B* series, the same method of recessive indicators was applied that was used in the case of homospermic androgenesis in *B. mori* (Astaurov, 1937, 1948; Ostriakova-Varshaver & Astaurov, 1947, &c.). The *mori* males used belonged to the strain homozygous for a recessive factor *ch* (*chocolate*) of the XIth chromosome determining a yellow-brown or red colour of larvae of the first instar instead of a black one. The true hybrids should have larvae of a dominant normal black colour and this, without exception, is actually observed in the control larvae hatched from unheated eggs. The appearance of exceptional red larvae from heated eggs laid by a *mandarina* female possessing no *chocolate* factor is possible only in cases of heterospermic androgenesis. In *B. mori* the males are the homogametic sex (*ZZ*), and each sperm introduces a *Z*-chromosome; androgenetic individuals should therefore develop into males. This is what actually takes place, without any exception, in the case of homospermic androgenesis. The paternal strain used in this series of experiments possessed two more distinct



TEXT-FIG. 2. Cytogenetical scheme of experiments on complete interspecific androgenesis in the cross ♀ *B.* (*Theophila*) *mandarina* × ♂ *B. mori* (type *T* × *B*).

recessive characters: white or *plain* caterpillars (chromosome II, *p*, *plain*) and *white moths*.

Three large series of experiments (M-3, M-5, and M-9) were made with the *T* × *B* type of cross. They produced 94,554 eggs from 344 interspecific crosses. The results are summarized in Tables 1 and 2.

TABLE 1

Heterospermic androgenesis of ♀ B. (Theophila) mandarina *cytoplasm with* ♂ B. mori *nucleus*

Experiment no. and crossing date	*Total no. females crossed and eggs laid*	*Heated 40° (exper.) or untreated (control)*	*Eggs*			*Developed up to larvae, but unhatched*					*Hatched larvae*			*Moving larvae extracted*
				%					% androg. of the number		Black (zygotic)			
			Total	*Hibernating*	*Non-diapause*	*Total*	*Black (zygotic)*	*Red (androg.)*	*of larvae*	*of eggs*	*Number*	*% hatching*	*Red (androg.)*	
						I. NON-DIAPAUSE EGGS								
M-3	112♀♀	Control	5,435	1	99	4,833	4,833	0	0	0	4,311	80·5	0	—
5–12.vii.1955	30,736	Exper.	25,301	0	100	1,136	91	1,045	92·5	4·1	76	0·3	1	69
M-5	72♀♀	Control	4,614	11·0	89·0	3,780	3,780	0	0	0	2,832	69·5	0	—
17–27.ix.1955	18,158	Exper.	13,544	0	100	820	57	763	93·0	5·6	50	0·4	0	41
I. TOTAL	184♀♀	Control	10,049	6·0	94·0	8,613	8,613	0	0	0	7,143	75·5	0	—
	48,894	Exper.	38,845	0	100	1,946	148	1,808	92·8	4·7	126	0·3	1	110
						II. DIAPAUSE (HIBERNATING) EGGS								
M-9	160♀♀	Control	12,223	100	0	—	—	—	—	—	7,775	63·5	0	—
5.ix–8.x.1955	45,660	Exper.	33,437	100	0	—	—	—	—	—	136	0·4	0	—
I+II TOTAL	344♀♀	Control	22,272	..	..	..	..	..	..	..	..	..	..	..
	94,554	Exper.	72,882	..	..	..	..	..	..	..	..	..	..	..

In the M-3 and M-5 series of experiments performed on non-hibernating eggs very similar results were obtained which make it possible to consider summary figures. Some peculiarities of the M-9 experiment are connected with the fact that it was performed on hibernating eggs.

In the M-3 and M-5 series taken together, about one-quarter of the eggs (10,049) obtained from 184 crosses were left without heat treatment (controls). The egg-shell of non-hibernating *mandarina* eggs is thin and transparent and

TABLE 2

Results of rearing the control and androgenetic larvae obtained in the experiment M-3

	Control (black)	*Androgenetic (red)*
1. Number of larvae reared (instar I)	280	69
2. Number and sex of caterpillars in instar V	unregistered	0 ♀+8 ♂ = 8
3. Number and sex of cocoons and imagos	102 ♀+110 ♂ = 212	0 ♀+5 ♂ = 5
Phenotype and genotype		
1. Sex	50% ♀♀ *ZW*+50% ♂♂ *ZZ*	All ♂♂ *ZZ*
2. Colour of just-hatched larvae	100% black *ch*/·	All red *ch ch*
3. Larval marking in instars II–V	100% wild-type (Mauricaud) p^M/p	All white *p*/*p*
4. Cocoon characters	Shape and size intermediate between *mandarina* and *mori*; all pointed; colour orange-yellow.	All have the shape of typical *mori*; not pointed; colour white and salmon-yellow as in paternal *mori* strain.
5. Moth characters	100% wild type, but a little lighter than in *mandarina* *Wm*/*wm*	All white as *mori* *wm*/*wm*

the colour of the larva seen through the shell can be established within 1 or 2 days before hatching. According to expectation, all the progeny developed from the control eggs showed without exception the regular dominant phenotype of true F_1 hybrids. All the 8,613 control F_1 larvae that developed to the stage of integument pigmentation were black, all the caterpillars (280) of the older instar showed a typical dominant *wild* or *Mauricaud* larval marking; all the cocoons (212) were of intermediate pointed form very characteristic of the F_1 hybrids and sharply different from that of *mori* and *mandarina* cocoons (Plate 1, fig. A). All the moths expressed the dominant dark colour, but it was a bit lighter than in *mandarina* (Plate 1, fig. B). The hybrids exhibited a normal 1 : 1 sex ratio (102 ♀♀ : 110 ♂♂).

A sharply different result was obtained in the experimental group of heated eggs. Only 1,946 eggs out of 38,845 (5 per cent.) developed to the stage of a pigmented unhatched larva; of them only 148 larvae were black and thus had a normal hybrid genotype. The main bulk—1,808 unhatched larvae or 92·8 per

cent.—were red, which indicated their androgenetic origin. Many larvae showed mobility and attempted to gnaw through the egg-shell. We expected an abundant output of androgenetic larvae, but only one proved to be capable of hatching without assistance.

This lack of vigour of the androgenetic larvae is obviously connected in part with their high homozygosity, resulting from self-fertilization. But a considerable part of this weakness should probably be ascribed to the incompatibility of the cytoplasm and the nucleus of different species, for in the case of *homospermic* androgenesis in *B. mori* the frequency of larval hatching is much greater.

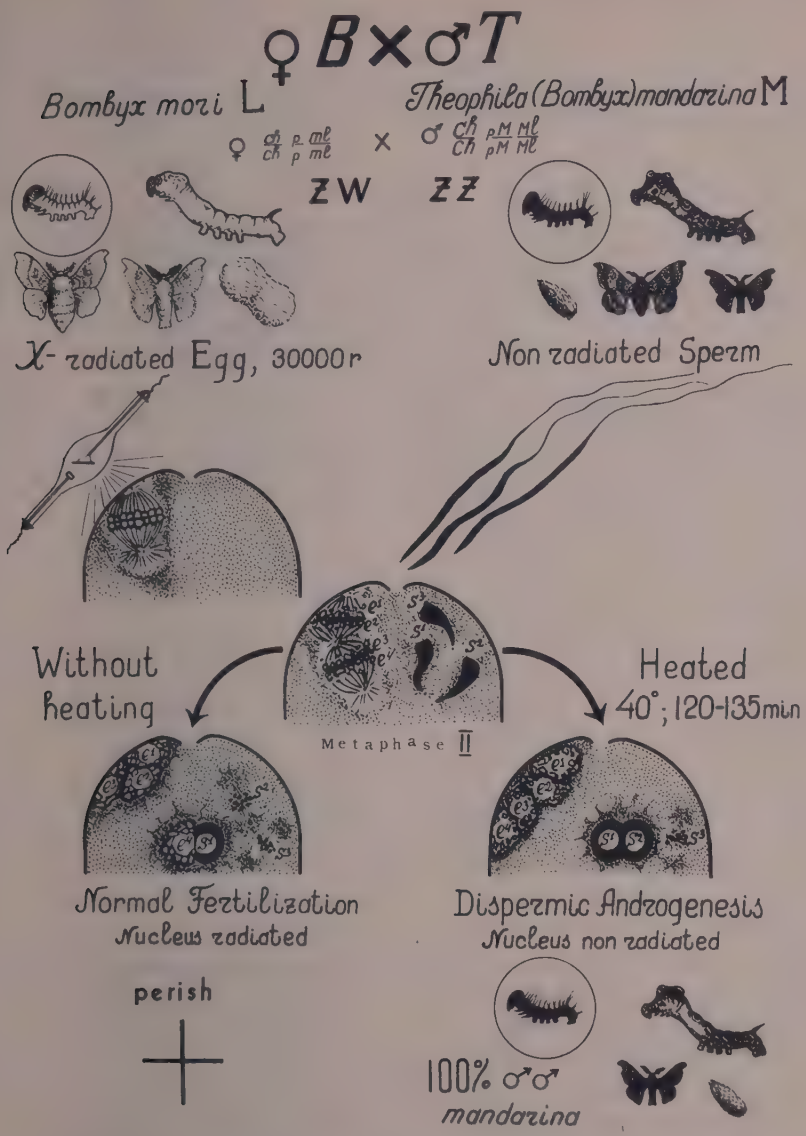
It is possible that the androgenetic larvae which, as it will be seen, were both genotypically and phenotypically *mori*, proved to be unadapted to gnaw through a solid *mandarina* egg-shell in which they were enclosed.

When it became clear that the androgenetic larvae were incapable of hatching, we had to dissect the egg-shells and to extract the larvae artificially. In such a way 110 red larvae, completely formed and motile, were extracted. Most of the unextracted androgenetic red larvae were in some respect underdeveloped, and incapable of movement.

The red larvae obtained from moths of the autumnal generation (experiment M-5) hatched late in autumn and could not therefore be reared. We tried to rear 69 caterpillars obtained in experiment M-3, performed on eggs of the spring generation. Many caterpillars proved of low viability and only 8 of them accomplished the first moulting. Probably their weakness was due in part to delayed extraction from the chorion, for their inability to hatch was quite unexpected and the aid we had to give them came therefore too late. If this is taken into account in future, heterosperm-androgenetic caterpillars may be reared more successfully. All eight red caterpillars which successfully performed the first moulting reached the Vth (last) instar; all of them, as was to be expected in the case of dispermic androgenesis, proved to be males and had a white patroclinic coloration (*pp*).

Five caterpillars spun cocoons and metamorphosed into five male moths. Both cocoons and moths had the typical habitus and coloration of the *mori* paternal line, though some of them were smaller than normal and underdeveloped (Plate 1, figs. A, B). Two males were back-crossed with the females (one of them with the two females) of the paternal strain and in all three families (703 progeny of one male and 330 of the other, 1,033 caterpillars in all) the offspring all proved to be typical representatives of the paternal *mori* species of the line used. They did not show the slightest signs of *mandarina* cytoplasmic influence.

The third, M-9, series of experiments of ♀ *mandarina* × ♂ *mori* type was performed on hibernating eggs. It was difficult therefore to observe the results of the experiments and to extract the formed androgenetic larvae, for the shell of hibernating eggs is opaque. Dissection of a test group of 449 eggs showed 31 or 6·9 per cent. of half-formed or fully developed red androgenetic larvae, so that as to the frequency and success of androgenesis the results obtained were in



TEXT-FIG. 3. Cytogenetical scheme of experiments on complete interspecific androgenesis in the cross ♀ *B. mori* × ♂ *B.* (*Theophila*) *mandarina* (type *B* × *T*; method of selective elimination of normal zygotic embryos by means of heavy X-raying).

principle the same as in the experiments considered above. We did not succeed in obtaining viable larvae from experiment M-9.

2. Let us now turn to the results obtained in reciprocal crosses of the $B \times T$ type: ♀ *B. mori* × ♂ *B. (Theophila) mandarina.*

The method of recessive indicators is not suitable here for early identification of heterosperm-androgenetic individuals, because in the *mandarina* silkworm

TABLE 3

Heterospermic androgenesis of X-rayed ♀ B. mori (*cytoplasm*) + ♂ B. (Theophila) mandarina (*nucleus*)

Experiment no. and crossing date	*Total of females*		*Heated 40° (exper.) or untreated (Control I, II)*	*Eggs*			*Larvae hatched*	
	X-rayed	*Crossed*		*Total*	*Reached pigmented serose stage*	*% of pigmented eggs*	*Number*	*Frequency*
M–2*a* 1–6.vii.1955	100	42	Exper.	13,351	?	?	1	—
			Control I	4,071	22	0·5	0	—
		12	Control II	5,476	?	?	0	—
M–2*b* 1–6.vii.1955	100	21	Exper.	6,564	..	..	2	—
			Control I	1,745	..	..	0	—
		25	Control II	17,920	..	..	0	—
M–4 8–10.vii.1955	90	80	Exper.	29,620	2,917	9·8	6	—
			Control I	5,621	135	2·4	0	—
M–6 16–19.ix.1955	100	26	Exper.	7,488	1,709	22·6	5	—
			Control I	5,127	22	16·0	0	—
		42	Control II	17,417	?	?	3	—
M–7 16–19.ix.1955	100	45	Exper.	14,288	2,946	20·6	14	—
			Control I	6,457	491	7·6	0	—
		39	Control II	20,974	?	?	2	—
M–2*a*, –2*b*, –4, –6, –7. TOTAL	490	214	Exper.	71,311	..	..	28	0·00039
			Control I	23,021	..	..	0	—
		118	Control II	57,767	..	..	5	0·00009
		332	Control I+II	80,788	..	..	5	0·00006

such signal genes have not yet been discovered. In this case use was made of a method of selective elimination of normal zygotic embryos with the aid of heavy X-raying (Text-fig. 3). Not long before coming out of the cocoon the *mori* parents were given an irradiation dose of about 30,000 r. This dose is sufficient to kill all the regular hybrid offspring, for they receive from the mother a chromosome set heavily damaged by X-rays. By contrast, as special experiments have shown (Astaurov, 1947 *a*, *b*), the offspring of androgenetic origin develop quite normally even when the maternal ooplasm has been given a gigantic irradiation dose of up to 540,000 r. These androgenetic individuals receive 'healthy' nuclear material only from their non-irradiated father. From the mother they inherit only the irradiated ooplasm. Judging by these experiments, the alterations occuring in the ooplasm under the influence of X-rays are practically harmless for the developing organism if the latter possesses an

unaffected diploid chromosome set. Therefore the homosperm-androgenetic progeny of silkworm develop normally, live, and produce offspring, even when they originated from an egg that had been irradiated with half a million r.

This result, which we think is of considerable interest for radiobiological theory, made it possible to hope that all the zygotic embryos in the experiments performed according to the plan just described would perish during embryogenesis so that all the viable larvae would be of heterosperm-androgenetic origin.

In five large series of experiments (M-2*a*, M-2*b*, M-4, M-6, M-7) the X-raying attained its purpose and practically all regular zygotes were killed. The results of these five series can be considered together. In Table 3 'Control I' are regular zygotic offspring of X-rayed *mori* females, obtained from the same interspecific crosses as the heat-treated eggs. 'Control II' are also zygotic offspring, but obtained from the X-rayed *mori* females which did not copulate with males of an alien species and were crossed with the males of the same *mori* strain. Both controls together in the five series comprise 80,788 X-rayed zygotes, of which only five eggs gave hatched larvae. It is quite possible that these sporadic cases of embryo survival are also due to androgenesis, occurring spontaneously.

In three series of experiments (M-4, M-6, and M-7) the number of eggs reaching the stage of serosa membrane pigmentation was noted. In all three the percentage of such eggs in the heat-treated group was greater than in the Control I group, which proves that heat treatment induces androgenetic development. In all three series together 8·4 per cent. of eggs were pigmented in the control and 14·7 per cent. in the experimental group, i.e. almost twice as many. Many of the embryos which developed to the stage of serosa pigmentation later reached the stage of a well-developed larva. From all five experimental series 28 heat-treated eggs gave rise to larvae which hatched without assistance. Though the absolute number of larvae is small, the frequency of larval hatching in the experimental group (0·00039) is 6·5 times greater than that in the control group (0·00006) and 39 times greater than the frequency of androgenetic *hatched* larvae in reciprocal crosses (0·00001). All this testifies to the probability that almost all of these 28 larvae are of heterosperm-androgenetic origin and this is also confirmed by the results of rearing them. Rearing wild silkworms, especially solitary and weakened androgenetic specimens, is very difficult and often ends in death from polyhedral disease. Thus it is not surprising that the success we obtained was rather modest, but nevertheless the results are quite definite.

Out of 28 larvae hatched in the experiments, 10 survived until the appearance of definitive caterpillar marking (IIIrd and IVth larval instars). The sex could be distinguished with confidence only in 5 specimens of which 4 proved to be males (Plate 2, figs. D, E, F, G) and one female (fig. H). Four specimens—3 males and 1 female—spun cocoons (Plate 2, fig. I). Metamorphosis was accomplished by one specimen and that one was a female.

The characters of the female cocoon and of the slightly underdeveloped female moth which emerged from it clearly resembled those of interspecific

hybrids. In all probability the appearance of this female represented one of the very rare cases of survival of zygotic offspring. All the other surviving specimens were typically *mandarina* in their body-size, larval marking, blood and cocoon colour, form and structure of cocoon, and general habitus. They were clearly distinguishable from interspecific hybrids (Plate 2, figs. D–G, I).

Thus, in the first adult heterosperm-androgenetic animals known to us no indications were found of a dependence of the hereditary transmission of specific characters on the maternal cytoplasm. Independently of the direction of interspecific crosses all the specific characters of larvae, pupae, and moths of androgenetic hybrids were obtained from the paternal species through the nuclear material of male sexual cells.

That specific characters are transmitted through the nuclear material is certainly not unexpected. But the fact that neither in heterosperm-androgenetic individuals nor in their offspring did we find any traces of transmission of specific characters through the maternal ooplasm seems to us of definite scientific significance. It shows that at least in this case of interspecific nucleo-cytoplasmic interaction the development of the cytoplasmic material in the course of embryogenesis is entirely subordinated to the influence of the genome.

SUMMARY

1. The obtaining of a complete interspecific androgenesis or merogony is justly considered an *experimentum crucis* for the solution of some basic questions pertaining to the so-called nucleus-cytoplasm problem. However, in spite of numerous attempts, adult heterosperm-androgenetic animals have not so far been obtained.

2. Successful induction of a complete intraspecific androgenesis in the domestic silkworm *B. mori* L. opens the possibility of obtaining heterospermic androgenesis in interspecific hybrids between the domestic silkworm and the wild one, *B. mandarina* (Moore).

3. It is shown that androgenetic hybrids originating from *mandarina* cytoplasm + *mori* nucleus can be obtained by means of strong heating of heterospermically inseminated eggs at the beginning of the meiotic metaphase II. Many androgenetic larvae ready to hatch were obtained, but only five adult androgenetic male moths. By back-crossing the heterospermic androgenetic males with females of the paternal species (*mori*) more than 1,300 individuals were obtained indistinguishable in every respect from those of the paternal species.

4. The reciprocal androgenetic hybrids originating from *mori* cytoplasm + *mandarina* nucleus can be produced by applying the same procedure but after a heavy pretreatment (30,000 r.) of the female gamete by X-rays, a method of selective elimination of the normal zygotic embryos. In this case several heterosperm-androgenetic caterpillars and cocoons were obtained but as yet no imagos.

5. In the first successful experiments in which adult interspecific androgenetic hybrids have been obtained, no indication was found of the control by the maternal cytoplasm of any character of the hybrids that distinguishes the parental species.

In this respect the data here reported are similar to the data, now very voluminous, obtained by interracial (intraspecific) androgenesis in *B. mori*. In the case of interspecific androgenesis, as in the interracial case, all the specific characters of larvae, pupae, and moths, irrespective of the direction of the interspecific cross, were received from the paternal species. In this instance at least, therefore, the specific differences are controlled by the nuclear material of the male sexual cells.

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EXPLANATION OF PLATES

Plate 1

Fig. A. Cocoons of 5 androgenetic males with *mandarina* cytoplasm + *mori* nucleus (right, below) with cocoons of the maternal *B. mandarina* (left, above) and the paternal *B. mori* (right, above) species and with the zygotic F_1 hybrids (left, below).

Fig. B. Androgenetic male moths *mandarina* (cytoplasm) + *mori* (nucleus) along with moths of the maternal *B. mandarina* and the paternal *B. mori* species as well as the zygotic F_1 hybrids (control). Experiment M-3. In each group left vertical row is of females, right vertical row is of males.

Plate 2

Fig. C. Photographs of *B. mandarina* (dark) and *B. mori* (light) moths, copulating in reciprocal combinations.

Figs. D, E, F, G. Photographs of four androgenetic male caterpillars obtained in the experiment M-7 (type $B \times T$).

Fig. H. A probably zygotic female caterpillar obtained in the same experiment.

Fig. I. Photographs of androgenetic cocoons obtained in the experiment M-7 of the $B \times T$ type (the upper row; excluding the extreme left cocoon belonging to a probably zygotic hybrid female) along with the cocoons of the maternal *B. mori* (left) and the paternal *B. mandarina* (right) species and with the control F_1 hybrids (bottom row).

(Manuscript received 12 : ii : 57)

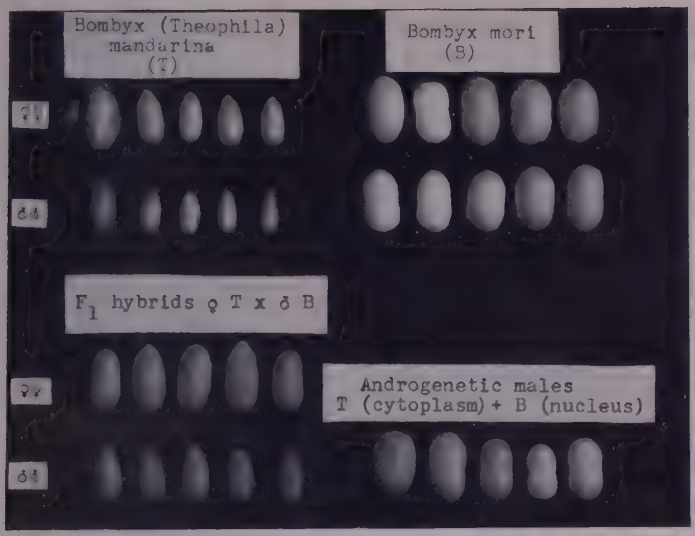


FIG. A

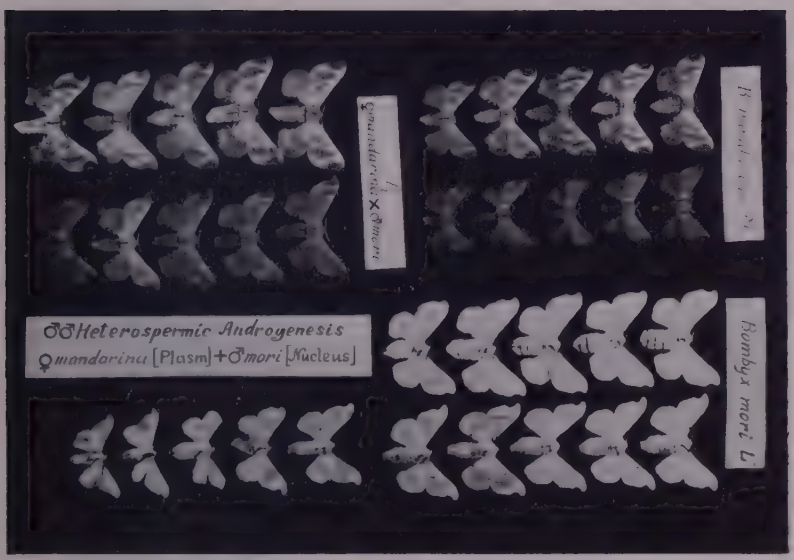
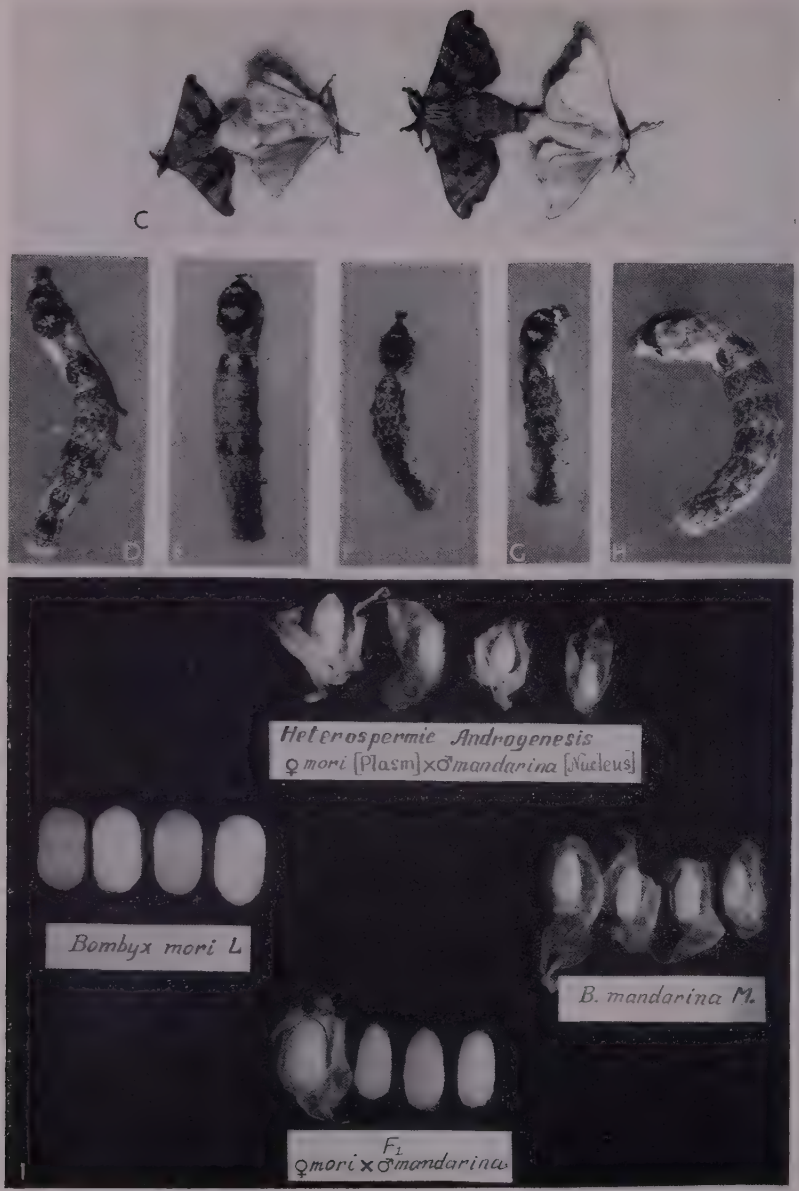


FIG. B

B. L. ASTAUROV *and* V. P. OSTRIAKOVA-VARSHAVER

Plate I



B. L. ASTAUROV *and* V. P. OSTRIAKOVA-VARSHAVER

Plate 2

Part 3, September 1957

Part 4, December 1957

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